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13. ABSTRACT *(Maximum 200 words)*

Urinary tract infections (UTIs), generally caused by Escherichia coli or Staphylococcus saprophyticus, are extremely common among young women. Although UTIs can be treated, we currently lack effective means to prevent frequently UTIs, which occur in 25% of women with first UTI. A necessary prerequisite to UTI is adherence of uropathogens to the vaginal and bladder epithelium. This report describes the second year of progress in a project whose overall goal is to define uropathogenic E. coli and S. saprophyticus-binding glycosphingolipids (GSLs) in the vaginal and bladder epithelium, shown in preliminary studies to function as bacterial receptors, as a prerequisite to the rational design of new agents that will prevent colonization and infection in women. Key progress includes: (1) greatly improving techniques for culturing and cryopreserving primary vaginal epithelial cells (2) broadening the scope and depth of studies related to vaginal epithelial cells; (3) development of several new methods, including a non-radioactive HPTLC immunostaining method and two new approaches for preparing a key GSL compound to be used in the next two years of funding; (4) demonstration of the importance of GSLs in mediating E. coli adherence to vaginal epithelial cells; and (5) publication of two related manuscripts.

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FOREWORD

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4. TABLE OF CONTENTS

5. INTRODUCTION

a. Overview

The overall purpose of this project is to investigate interactions between bacteria which commonly cause urinary tract infection (UTI) and their cognate host cell receptors in the vaginal and bladder epithelium in order to design novel, non-antibiotic methods for preventing UTIs. The project is focused on studying the two most common uropathogens causing UTI in young women, Escherichia coli and Staphylococcus saprophyticus, as well as their interactions with glycosphingolipids (GSLs) on the cell surface of the bladder and vagina. In the first two years of this project, we will define the key GSLs on the eukaryotic cell surface that uropathogenic bacteria use for attachment and then in the last two years, we will take advantage of new biochemical techniques using carbohydrate mimetics to design UTI prevention methods that avoid the induction of antimicrobial resistance. This report describes progress made in the second of four years of this grant.

b. Background

Acute uncomplicated UTIs caused by E. coli and S. saprophyticus occur in an estimated 7 million young women each year at an annual cost for diagnosis and treatment exceeding one billion dollars. Over half of all women have had a bacterial UTI by their late 20's and approximately 20% of women with UTI suffer very frequent ($\geq$ 3/year) recurrences (1). Nonetheless, the only currently available preventive modality for these recurrent infections is antimicrobial prophylaxis. Though effective, antimicrobial prophylaxis may promote the emergence of drug-resistant strains (2). In addition, women typically revert to having frequent recurrences once prophylaxis ceases and little is known about why some women suffer frequent recurrences of UTI, since this phenomenon cannot usually be explained by underlying functional or anatomic abnormalities of the urinary tract (2). The interaction of infecting bacterial strains with the women's epithelial cells appears to be a critical point in the infectious process that determines host susceptibility, in particular the availability and nature of host cell bacterial ligands such as GSLs (3-5).

GSLs are important components of the glycocalyx surrounding mammalian cells and consist of an oligosaccharide moiety exposed on the cell surface, to which organisms attach, covalently linked to a lipid portion embedded in the outer leaflet of the plasma membrane. They serve as eukaryotic cell adhesion sites for many pathogens and their toxins, including E. coli, Pseudomonas aeruginosa, Helicobacter pylori, HIV, parvovirus, rotavirus, cholera toxin, verotoxin of E. coli 0157, and others (6-10). Based on the structures of their carbohydrate components, they are grouped into families, such as the lacto- and neolacto-series, the globoseries, and the ganglioseries GSLs. GSLs are synthesized by the sequential action of glycosyltransferases, many of which are tissue-specific and/or genetically determined (11). Thus, GSLs on the cell surface play an important role both in determining tissue tropism and an individual host's susceptibility to specific infectious diseases

Among uropathogenic E. coli, isolates expressing the pap -encoded family of adhesins are significantly overrepresented among strains collected from patients with UTI as compared with fecal isolates from patients without UTI (12). The GSL receptors for these adhesins in the kidney are the globoseries GSL family that contain a minimal receptor consisting of a

galactose α-1-4 galactose moiety (12). Although $\underline{S}$. saprophyticus is the second most common cause of UTI, to our knowledge, we are the first to actually investigate whether it binds to GSLs. In our grant proposal, we showed preliminary data demonstrating that the wild-type $\underline{S}$. saprophyticus strain ST352 binds asialo GM1 (ASGM1), a neutral ganglioseries GSL and that other wild-type $\underline{S}$. saprophyticus isolates bind to ASGM1 and/or structurally-related ganglioseries GSLs. Paradoxically, while globoseries GSLs have been identified in kidney tissue and vaginal epithelium, the bladder has been little studied with respect to GSLs, despite the fact that it is the most common site of UTI. Previous studies of GSLs in native bladder tissues have focused on oncogenesis or development and have not included GSLs that are directly involved in adhesion of uropathogens (ganglioseries and globoseries GSLs). Of note, other urogenital pathogens, including $\underline{C}$. albicans, $\underline{C}$. trachomatis, $\underline{N}$. gonorrhoeae, have also been reported to bind to ASGM-1 and it is possible that a single class of inhibitors could prevent adherence and infection with all of these agents.

c. Brief summary of preliminary data presented in original proposal

Our original proposal presented preliminary data demonstrating that primary cultures of human bladder epithelial cells are a promising model system for the study of bladder GSLs in the pathogenesis of UTI caused by $\underline{E}$. coli or $\underline{S}$. saprophyticus. Specifically, we showed that these cell cultures appear to express globoseries GSLs, the host cell binding ligand for an important class of uropathogenic $\underline{E}$. coli, those expressing pap-encoded adhesins. We previously showed that SGG and other globoseries GSLs are surface exposed in human kidney tissues and exfoliated vaginal epithelial cells (3). In our preliminary immunocytology experiments with primary cultures of human uroepithelium, the cells demonstrated bright immunofluorescent staining with MAb ID4 directed against SGG, suggested that this epitope is also surface exposed on these bladder cells. We also showed that $\underline{S}$. saprophyticus, the second most common cause of UTI in young women, binds to ganglioseries GSLs, especially ASGM1 and ASGM2. In addition, we demonstrated the presence of ASGM1 among GSLs extracted from human kidney and the surface exposure of this epitope in kidney sections in specific histological areas where bacteria also adhere. Another ganglioseries GSL, GM1, was identified among GSLs extracted from human kidney tissues and vaginal epithelial cells. ASGM1 appears to be surface exposed on both kidney and cultured primary bladder cell surfaces, as shown by positive immunofluorescent staining with MAb TKH-7, directed against ASGM1.

d. Originally proposed hypotheses

The original hypotheses of this project have been supported by data obtained during the first two years of the grant and thus remain unchanged, as listed below. The overall goal of this project remains to define the key eukaryotic cell surface GSLs that are used by uropathogenic bacteria for attachment and then to take advantage of new biochemical techniques utilizing carbohydrate mimetics to design novel means for preventing UTIs that avoid the use of antimicrobials.

(1) We hypothesize that globoseries and ganglioseries GSLs are present in primary cultures of bladder transitional epithelium and vaginal epithelium and serve as binding sites for $\underline{E}$. coli and $\underline{S}$. saprophyticus, respectively.

(2) We hypothesize that the GSLs identified in the first hypothesis are surface exposed in primary cultures of bladder transitional epithelium and vaginal epithelium and are functionally relevant for E. coli and S. saprophyticus attachment and infection.

(3) We hypothesize that carbohydrate mimetic and synthesis techniques can be used to design high-affinity inhibitors of E. coli and S. saprophyticus binding to vaginal and bladder transitional epithelium.

e. Original technical objectives

Overall, our original technical objectives remain unchanged as we complete the second year of the project. This past year, we have further developed some of the unique collaborative opportunities that arose during the first year of funding. In addition, we have begun using two other methodologies directed towards purifying larger amounts of the key compound needed for the third technical objective. Lastly, in the past year, one of our collaborators (Dr. Toyokuni) has obtained a senior faculty at the University of California Los Angeles. This will result in an improved access to state-of-the-art synthetic organic chemistry facilities and support for the accomplishment of Technical Objective 3. These changes are discussed in greater detail below. Our technical objectives are as follows:

(1) We will extract and characterize GSLs that bind E. coli or S. saprophyticus from primary cultures of bladder transitional epithelium and vaginal epithelium, according to the following sequence: (a) purify the GSLs using high-performance liquid chromatography (HPLC); (b) identify bacteria-binding GSLs by overlaying radiolabeled isolates of E. coli and S. saprophyticus on these GSLs separated on high-performance thin-layer chromatography (HPTLC); (c) confirm the identities of these GSLs using specific monoclonal antibodies (MAbs) directed against the GSLs in HPTLC immunostaining assays; and (d) perform carbohydrate structural analysis on the bacteria-binding GSLs.

(2) To demonstrate that the GSLs identified in Hypothesis 1 are surface exposed in primary cultures of bladder transitional epithelium and vaginal epithelium and are functionally relevant for E. coli and S. saprophyticus attachment and infection, we will: (a) test representative isolates for adherence to primary bladder cell and vaginal epithelial cultures; (b) utilize immunofluorescence staining of the same cell cultures with MAbs directed against relevant GSLs; (c) repeat 2a and 2b after pretreatment of the cell cultures with an inhibitor of GSL receptor synthesis; and (d) repeat 2a after pretreatment of the cell cultures with the MAbs directed against relevant GSLs.

(3) We will use carbohydrate mimetic techniques to design inhibitors of bacterial adherence, focusing initially on the interaction of E. coli with sialosyl galactosyl globoside (SGG, a GSL to which E. coli binds with high affinity; see preliminary data). We will test the inhibitory efficacy of the compounds in bacterial overlay assays and in bacterial adherence assays, as described in the second objective.

6. BODY OF REPORT

A. Overview

1. Review of outcome of changes and opportunities occurring in the first year of funding:

(1) Movement of Dr. Hakomori's laboratory to the Pacific Northwest Research Foundation in Seattle:

Dr. Hakomori and members of his group have remained fully involved in their collaborative and consultative roles in this project, as evidenced by one of our publications this year.

(2) Movement of Dr. Stroud to the Molecular Medicine Department at Northwest Hospital in Seattle:

Dr. Stroud joined an oncology-related carbohydrate research group located in Northwest Hospital, headed by Dr. Eric Holmes, an expert in glycosyltransferases. This move has had a very positive impact on our project. Through training and experience received in this environment and through access to specialized equipment, reagents, and cell lines available there, Dr. Stroud has broadened his approach to several of the technical objectives of the project.

(3) Establishment of a new collaboration with Dr. Steven Levery of the Complex Carbohydrate Research Center (CCRC) of The University of Georgia, Athens:

We are continuing this fruitful collaboration, which has resulted in a recently accepted publication in Biochemistry and ongoing structural studies of additional compounds isolated from human urinary tract tissue (kidney).

(4) Establishment of a collaboration with Drs. M. Juliana McElrath and Florian Hladik of the Fred Hutchison Cancer Research Institute and the University of Washington:

This collaboration has also continued and we have expanded it to include our group as qualified individuals authorized to obtain vaginal tissues under our collaborators' institutional review board approval mechanisms. This has resulted in our establishing a number of vaginal epithelial cell lines from individual patients (see results).

2. New opportunities and approaches during the second year of funding:

1. Vaginal epithelial cell cultures:

We expanded the scope of our involvement with our new collaborators in this aspect of the project so that now we are independently and more regularly acquiring tissues for the establishment of new cell lines. This has increased the amount of material available to us and greatly assisted our progress.

2. New approaches taken by Dr. Stroud:

In order to prepare for the tasks delineated in Technical Objective 3, Dr. Stroud has been pursuing two separate methods to generate relatively large quantities of sialosyl galactosyl globoside (SGG) for chemical modification studies. These are detailed below.

3. Meetings with collaborators based outside Seattle:

a. Dr. Atala: Dr. Stapleton met with Dr. Atala in April 1998 at the International Bladder Research Congress, where she presented a talk entitled, "Host factors in susceptibility to urinary tract infection". She met with Dr. Atala again in May 1998 visited his laboratory in July.

b. Dr. Levery: Dr. Stroud met with Dr. Levery in July 1998 in Seattle. Drs. Stapleton and Stroud both met with him in August while attending the XIX International Carbohydrate Symposium. At this meeting, we presented a poster in collaboration with Dr. Levery.

c. Dr. Toyokuni: Drs. Stapleton and Stroud also met with Dr. Toyokuni in August while attending the XIX International Carbohydrate Symposium in August 1998, where logistics and advantages of working with him in his new position at UCLA were discussed, as well as the synthetic strategies to be pursued in the next funding period.

B. Original Statement of Work

The original technical objectives set for all four years of funding are listed below. Tasks on which we have made progress are noted in bold italic.

Technical Objective 1:   Extract and characterize GSLs that bind E. coli or S. saprophyticus from primary cultures of bladder transitional and vaginal epithelium.

Task 1:	*Months 1 to 6: cultivation of primary cultures of bladder and vaginal epithelial cells*
Task 2:	*Months 7 to 12: extraction of GSLs from bladder and vaginal cell cultures*
Task 3:	*Months 7 to 12: bacterial overlay assays*
Task 4:	*Months 7 to 12: immunostaining assays*
Task 5:	*Months 13 to 24: carbohydrate structural analysis*
Task 6:	*Months 25 to 36: data analysis and publication*

Technical Objective 2:   Demonstrate that the GSLs identified in Technical Objective 1 are surface exposed in primary cultures of bladder transitional epithelium and vaginal epithelium and are functionally relevant for E. coli and S. saprophyticus attachment and infection.

Task 1:	***Months 18 to 30: bacterial adherence assays to test representative isolates for adherence to primary bladder cell and vaginal epithelial cultures***
Task 2:	***Months 18 to 30: immunocytology procedures utilizing immunofluorescence staining of the same cell cultures with MAbs directed against relevant GSLs***
Task 3:	***Months 24 to 36: PDMP treatment of cell cultures, followed by GSL extraction and quantification and bacterial adherence assays***
Task 4:	Months 24 to 36: MAb pre-treatment, followed by GSL extraction and quantification and bacterial adherence assays
Task 5:	Months 30 to 36: data analysis and publication

Technical Objective 3: Use carbohydrate mimetic techniques to design inhibitors of bacteria adherence, focusing initially on the interaction of E. coli with SGG; test the inhibitory efficacy of the compounds in bacterial overlay assays and in bacterial adherence assays.

Task 1:	***Months 24 to 42: synthesis of linear mimetic compounds***
Task 2:	Months 27 to 42: structural analysis of resulting compounds (procedures to begin as each new compound is prepared)
Task 3:	Months 36 to 48: testing of inhibitory capacity of linear mimetic compounds in bacterial overlay assays, focusing on SGG as the target GSL to which binding should be inhibited
Task 4:	Months 36 to 48: testing of inhibitory capacity of linear mimetic compounds in bacterial adherence assays, using primary cultures of bladder and vaginal epithelial cells
Task 5:	Months 30 to 48: synthesis of multivalent structures from linear structures that are effective in Tasks 3 and 4
Task 6:	Months 40 to 48: repeating Tasks 3 and 4 using multivalent compounds
Task 7:	Months 40 to 48: data analysis and publication

C. Details of Progress

1. Technical Objective 1

a. Task 1, Months 1 to 6: cultivation of primary cultures of bladder and vaginal epithelial cells

1. Experimental methods, assumptions and procedure

Primary cultures of human bladder epithelial cells were provided by Dr. Anthony Atala and maintained in serum free keratinocyte media using standard tissue culture techniques, as in our preliminary studies and as he has described (13). Briefly, cells were maintained in serum- and antibiotic-free keratinocyte medium and passed at 70% confluence in a ratio of 1:4 or 1:6 (13). Each time cells were passed, the number of flasks were maximally expanded to prepare cells for the purification of GSLs of interest. This procedure was continued with each cell sample until senescence was noted, usually at about passage 12.

During the first year of funding, primary cultures of vaginal epithelial cells were established de novo in our laboratory through the collaboration with Drs. McElrath and Hladik. This past year, this collaboration has enabled us to begin obtaining specimens primarily as well as working with specimens obtained and processed by Dr. Hladik. Thus, much of our primary cell culture efforts during this past year were directed towards optimizing our culture techniques for vaginal cell lines and maximally expanding these lines to collect material for GSL purification. As with our primary bladder epithelial cells, cultures were grown until senescence was observed, usually about 5 to 6 passages. Cell lines with fibroblast contamination were discarded.

2. Results and discussion

Primary bladder epithelial cells: Cultivation of bladder epithelial cells is an established technique in our hands now. During the first funding year, we cultivated 250 flasks of cells in one 4 month period to harvest 4 ml of cells (225 cm^2 flasks) as pellets and for GSL extraction. However, it was important that we further develop our vaginal epithelial cell culture techniques Thus, during the second year of funding, we maintained our bladder epithelial cell lines at a level time somewhat lower than that of the vaginal epithelial cell lines.

Primary vaginal epithelial cells: A major effort was made to improve culture techniques to maximize survival and success of this effort. To date, we have established 20 lineages of cells. We focused on refining techniques for establishing primary vaginal epithelial cell lines from tissue samples, as follows:

a. We tried numerous techniques for minimizing the survival of fibroblasts, such as varying the media used at various early stages of establishment. We adapted an immunocytochemical assay for staining cells grown with MAbs which we had previously used for bladder epithelial cells and stained with one MAb directed against human epithelial cells and another against human epithelial cells and documented that at present, fibroblast contamination of our cultures is effectively zero. We are now unable to identify fibroblasts in ongoing vaginal epithelial cell lines visually or using specific MAbs.

b. By experimenting with various methods of preparing the initial tissue sample for seeding flasks, we decreased the amount of time needed for cells newly prepared from tissues to attach to the flasks, from weeks to days.

c. We maximized our expansion capabilities by careful observation of the characteristics of the cells during all phases of culture and by adapting our methods to large surface area flasks and plates. We are now able to maintain a visually normal phenotype through passage 7.

d. Our collection of cryopreserved cells has been greatly augmented. We have vials representing 11 different lines currently frozen in liquid nitrogen.

e. We performed some preliminary investigations of the use of in vitro epithelial cell differentiation techniques on the light microscopic morphology and adherence characteristics of the cells (see below). Through one of our collaborations in an NIH-sponsored clinical study of the effects of various exposures on normal vaginal physiology, we have access to the advice of a expert in the histopathology of gynecological tissues. This collaboration has helped us to assess the morphology of our cultured cells in comparison to the various layers present in the many vaginal biopsies from normal young women that have been examined through this study.

We encountered relatively few problems in the cultivation of vaginal epithelial cells this year, as follows:

a. We tried a new characterized keratinocyte medium offered by the same manufacturer that supplies the media we used for both bladder and vaginal epithelial cells. The new media had minor negative effects on the bladder cells but caused a considerable reduction in cell viability and expansion for the vaginal epithelial cells. This has been discontinued.

b. Time constraints related to use of the biological safety hood and inadequate incubator space: After encountering limitations related to these constraints, we adjusted personnel work hours to allow continuous use of the hood and borrowed incubator space from a collaborator. However, since this individual's laboratory is located nine floors away in another wing, we are presently evaluating bids for a new incubator, which we will purchase in the next funding year.

3. Recommendations in relation to the Statement of Work

1. Primary bladder epithelial cells: We plan to continue to cultivate bladder cell lines throughout the third year of funding, as needed for further purification of GSLs.

2. Primary vaginal epithelial cells: In the next funding period, we will continue to establish new primary vaginal epithelial cell lines as well as to expand lines already established and cryopreserved. We will continue to work to extend the time of cultivation for each line. During the past year of funding, we planned to study the effects of estrogen and progesterone (separately and together) on the length of time that cells can be cultivated and the ability to expand cells. However, we postponed these studies until we could obtain phenol red free medium, since phenol red is known to have weak estrogenic effects in some estrogen-responsive tissues. This was special ordered and recently arrived, allowing us to begin

these studies. If the results of hormone treatments are successful with respect to simple parameters such as growth rate, we will expand these studies beyond the merely descriptive and practical to explore other effects of treating the cells with these hormones, such as their susceptibility to bacterial attachment, etc. Knowledge gained through the above-mentioned collaborative (NIH-sponsored) clinical study of vaginal physiology will be applied to our in vitro system as deemed appropriate (relevant data from this study are currently being analyzed).

b. Task 2, Months 7 to 12: extraction of GSLs from bladder and vaginal cell cultures

1. Experimental methods, assumptions and procedure

Extraction of GSLs from primary cultures of human bladder epithelial cells and vaginal epithelial cells was performed as in our preliminary data, our previous work using exfoliated vaginal epithelial cells (3), and in the work performed during the first year of funding. At each step, GSLs were chromatographed in various organic solvent systems and stained with orcinol (carbohydrate detection stain) to assess the purity of individual bands. In addition, HPTLC bacterial overlay procedures as described below were performed to identify and monitor the purification of GSLs of interest. Briefly, the GSL isolation and purification steps are as follows: cell cultures were maintained as described (13) then cells were trypsinized, pelleted, and washed, and the total GSLs were obtained by extracting the pellets with 10 volumes isopropanol:hexane:water (IHW; 55:25:20 by volume) with sonication in a warm bath and centrifugation at 2,500 RPM for 10 minutes. Some samples were subjected to a modified Folch procedure to obtain the upper and lower phase GSLs (15). Samples for which it was appropriate to separate neutral GSLs from gangliosides had the upper phase GSLs applied to reverse phase column chromatography, followed by anion exchange chromatography (15).

2. Results and discussion

To date, we have prepared cell pellets of at least 0.5 ml obtained from 8 samples of vaginal epithelial cells and 7 samples from bladder epithelial cells). As pellets were amassed in sufficient quantity, GSLs were extracted. Thus, these samples are in various states of GSL purification, depending on total volume; all samples are in a sufficiently developed stage to perform immunostaining assays and/or bacterial overlay assays. Because of some preliminary findings we noted regarding bacterial adherence to vaginal epithelial cells that have been differentiated morphologically in culture (see below), we have begun to investigate potential differences in GSL expression when undifferentiated and differentiated cells are compared. Thus, 5 of our 6 vaginal epithelial cell pellet samples that are in the process of GSL purification consist of paired differentiated and undifferentiated samples from the same cell lines. For those samples where sufficient material is present, further purification steps will include preparative HPTLC (16) and/or high-performance liquid chromatography (17), focusing on GSLs that bind E. coli or S. saprophyticus.

The rotoevaporator that we purchased during the last funding period for use in the University of Washington laboratory to augment the instrumentation available in Dr. Stroud's laboratory has improved the efficiency of drying and purifying GSLs. As we predicted, this equipment has helped the two laboratories to simultaneously carry out complementary

purification steps. In addition, we have developed a routine of scanning and exchanging new HPTLC data via FTP protocols to facilitate rapid communication between the laboratories of Drs. Stapleton and Stroud.

Preliminary data from samples of vaginal epithelial cell subjected to some of the GSL purification steps described above is shown in Figure 1. Yields from these studies have improved over our earlier attempts in the first year of funding, in part because survival of the cell cultures has improved and we are able to obtain substantially more material.
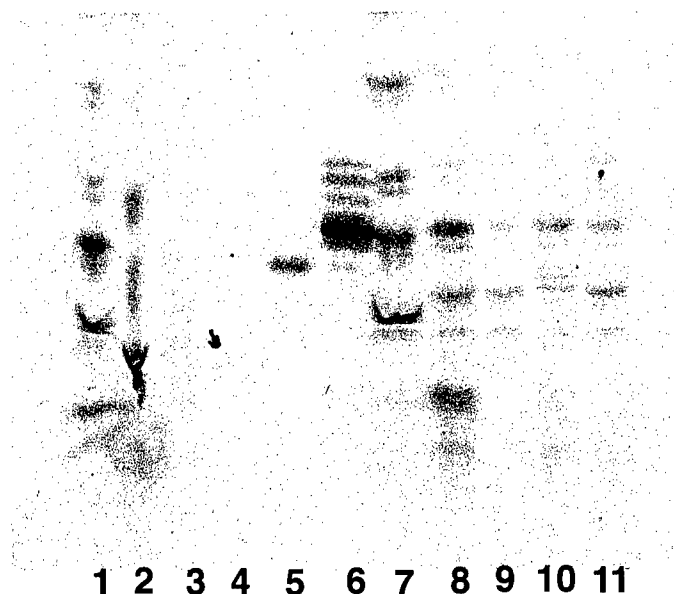

Figure 1. Orcinol stained thin-layer chromatography plate

Orcinol stained HPTLC plate showing partially purified total GSL preparations from four different cell primary vaginal epithelial cultures along with authentic GSL standards. GSLs were separated on HPTLC plates, then stained with the orcinol reagent to visualize carbohydrate **Lane 1:** GSLs extracted from V17 vaginal epithelial cell cultures, differentiated; **Lane 2:** GSLs extracted from V17 vaginal epithelial cell cultures, undifferentiated; **Lane 3:** GSLs extracted from V17 vaginal epithelial cell cultures that were pre-treated with an inhibitor of GSL synthesis (PPPP); **Lane 4:** standard sialosyl galactosyl globoside (SGG) from human kidney; **Lane 5:** standard globoside (Gb4) from human erythrocytes; **Lane 6:** standard ceramide trihexosyl (CTH) from human erythrocytes; **Lane 7:** GSLs extracted from V10 vaginal epithelial cell cultures, differentiated; **Lane 8:** GSLs extracted from V9 vaginal epithelial cell cultures, differentiated; **Lane 9:** GSLs extracted from V9 vaginal epithelial cell cultures, undifferentiated; **Lane 10: :** GSLs extracted from V5 vaginal epithelial cell cultures, differentiated; **Lane 11:** GSLs extracted from V5 vaginal epithelial cell cultures, undifferentiated. Note that SGG and globoside standards (lanes 4 and 5) are difficult to see in this image because of slight underloading, but largely because of the imaging techniques used for this scan. Samples from the V17 cell lines have not

undergone reversed phase column chromatography, accounting for the smeared appearance of bands in these lanes.

An example of GSL samples prepared from primary vaginal epithelial cell cultures and subjected to further purification steps is shown in Figure 2 below:
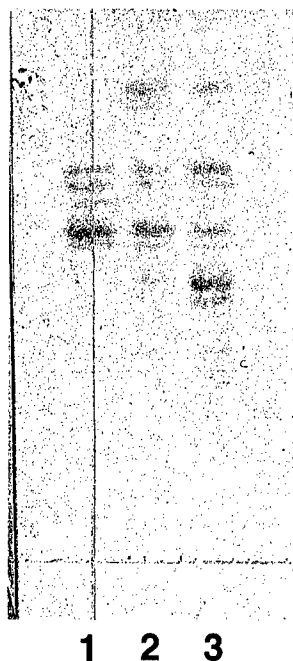

1   2   3

Figure 2. Orcinol stained thin-layer chromatography plate

Orcinol stained TLC plate showing examples of GSL containing fractions from primary cultured vaginal epithelial cells after further purification steps (reversed phase column chromatography). GSLs were separated on HPTLC plates, then stained with the orcinol reagent to visualize carbohydrate. **Lane 1**: CTH standard from human erythrocytes; **Lane 2**: GSLs extracted from V17 vaginal epithelial cell cultures, differentiated; **Lane 3**: GSLs extracted from V17 vaginal epithelial cell cultures, undifferentiated.

3. Recommendations in relation to the Statement of Work

1. We will continue the GSL purification steps remaining for each of our bladder and vaginal epithelial cell samples in a logical and efficient order that will maximize yields. At the XIX International Carbohydrate Symposium, we saw interesting data derived from newer methods of carbohydrate structural characterization. As described below, we will work with Dr. Levery to apply some of these methods, such as electrospray mass spectroscopy, can be applied to our samples,. Such alternative methods will decrease the amount of cell culture resources applied to this technical objective, freeing our time and effort for moving forward with later objectives.

2. We will continue to explore standard tissue culture methods to extend the period of time during which normal primary vaginal epithelial cells can be maintained but will focus on whether estrogen and/or progesterone have any effect on viability and the maintenance of normal morphology. Also, we will expand the number of cell lines for which we have prepared paired undifferentiated and differentiated samples.

3. We will likely purchase a scanner for Dr. Stapleton's laboratory, since the University of Washington core computer facility we have been using is now charging per hour of use. We already have appropriate software available and can integrate our efforts with software and equipment available in Dr. Stroud's facility.

c. Task 3, Months 7 to 12: bacterial overlay assays

1. Experimental methods, assumptions and procedure

This assay involves separating GSLs on HPTLC plates and overlaying the plates with radiolabeled bacteria (3, 18). E. coli organisms were metabolically labeled with [^{35}S]methionine as previously described (3). S. saprophyticus organisms were also metabolically labeled with [^{35}S]methionine using a gonococcal labeling method (Mandrell, unpublished data) that we adapted for S. saprophyticus (Stapleton et al., manuscript in preparation). The organisms have a final specific activity of 0.01 cpm/organism, similar to the specific activity achieved using comparable methods for E. coli (3). Radioactive bacterial overlay assays were performed as previously described, with a HPTLC plate run in parallel for orcinol staining. For the bacterial overlay assays with E. coli, we have continued to primarily use metabolically labeled wild type E. coli R45 (3), which expresses the pap-encoded class II adhesin (19) and thus specifically recognizes globoseries GSLs. However, we have performed some assays that confirmed expected GSL binding patterns of organisms expressing Class II or III pap-encoded adhesins (4). Overlays with S. saprophyticus have been performed using wild type isolate ST352, as in our preliminary data.

2. Results and discussion

Examples of bacterial overlay assays performed using the binding of E. coli expressing pap-encoded adhesins to assess the identities of GSLs among the compounds isolated from primary vaginal epithelial cells are shown in Figures 3 and 4 below. GSL preparations are in differing stages of purification, as indicated.
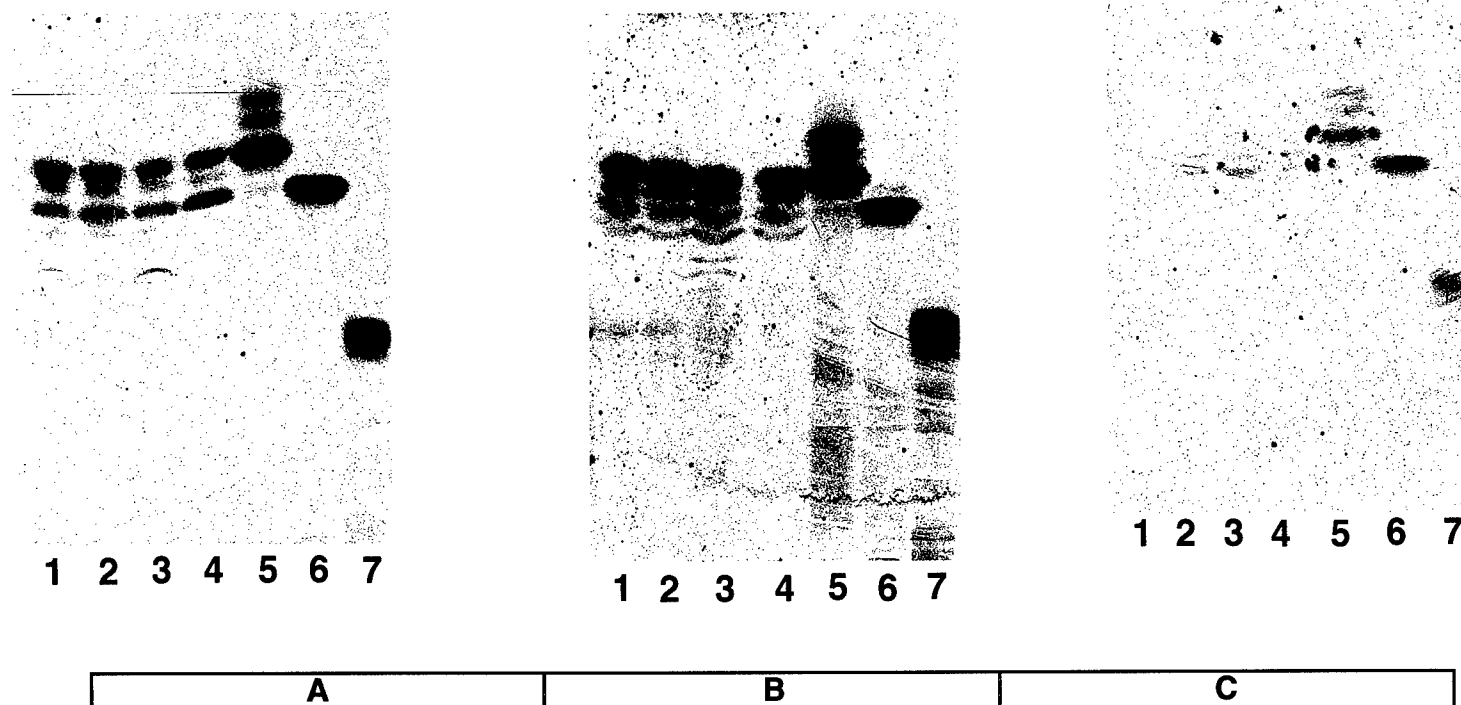
Figure 3. Binding of E. coli expressing pap-encoded adhesins to glycosphingolipids partially purified from human primary vaginal epithelial cultures

Primary vaginal epithelial cells were grown to 90% confluence, harvested, and counted in a hemocytometer. Total organic extraction in isopropanol:hexane:water (55:25:20 vol:vol) as well as reverse phase column chromatography were performed. Samples were separated on HPTLC plates and plates were then overlaid with metabolically [^{35}S]methionine-labeled E. coli. **Panel A**: E. coli R45, a wild type UTI isolate expressing a Class II pap-encoded adhesin; **panel B**: E. coli pDC-1/HB101, a recombinant isolate expressing solely a Class II pap-encoded adhesin; **panel C**: E. coli F24, a wild type UTI isolate expressing Class III pap-encoded adhesin. Autoradiographs are shown. **Lane 1**: GSLs extracted from V5 vaginal epithelial cells; **Lane 2**: GSLs extracted from V6 vaginal epithelial cells; **Lane 3**: GSLs extracted from V8 vaginal epithelial cells; **Lane 4**: GSLs extracted from V9 vaginal epithelial cells; **Lane 5**: ceramide trihexaosyl (CTH; globotriaosyl ceramide; Gb3) standard from human erythrocytes; **Lane 6**: globoside (Gb4) standard from human erythrocytes; **Lane 7**: sialosyl galactosyl globoside (SGG) from human kidney. A fourth plate, not shown, was overlaid with a uropathogenic E. coli without pap-encoded adhesins and produced a blank autoradiograph.
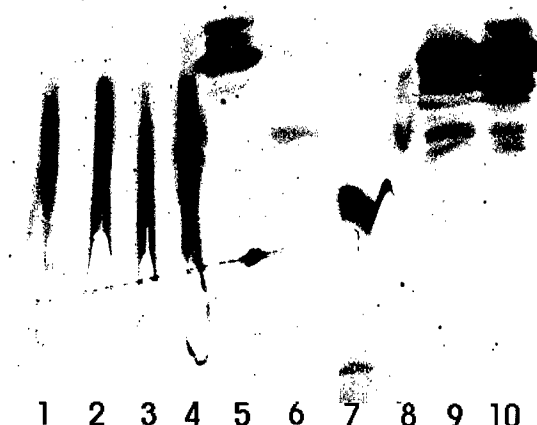
1 2 3 4 5 6 7 8 9 10

Figure 4. Binding of E. coli R45 to crude GSL fractions and GSLs partially purified from human primary vaginal epithelial cultures

Primary vaginal epithelial cells were grown to 90% confluence harvested, and counted in a hemocytometer. Total organic extraction in isopropanol:hexane:water 55:25:20 vol:vol followed by reverse phase column chromatography for some samples were performed. Samples were separated on HPTLC plates and plates were then overlaid with metabolically [^{35}S]methionine-labeled E. coli R45. **Lanes 1 and 2:** crude preparation of GSLs extracted from V5 vaginal epithelial cells; **Lanes 3 and 4:** crude preparation of GSLs extracted from V9 vaginal epithelial cells; **Lane 5:** standard CTH from human erythrocytes; **Lane 6:** galactosyl globoside standard prepared from human kidney; **Lane 7:** SGG standard from human kidney; **Lane 8:** GSLs extracted from V17 vaginal epithelial cell cultures that were pre-treated with an inhibitor of GSL synthesis (PPPP); **Lanes 9 and 10:** GSLs extracted from V17 vaginal epithelial cells and subjected to reverse phase column chromatography.

Our preliminary data and work done in the first funding period performed using extracts of GSLs from primary cultures of bladder epithelial cells and from primary vaginal epithelial cells demonstrated the presence of globoseries GSLs in a sample of material from a limited number of individuals. During the second year of funding, our collaborative opportunities for obtaining vaginal tissue samples were expanded and we were substantially more successful in perpetuating and expanding our cell lines. Thus, we focused our efforts with respect to Task 3 towards performing parallel bacterial overlay assays on samples from a larger number of individuals. In addition, we focused on studying GSL samples that represented cell lines that were either already sufficiently expanded so that relatively large amounts of GSLs could be extracted for parallel bacterial overlays, immunostaining assays and further GSL purification of material from the same individual; or, alternatively, we worked with lines that represented very successful clones from which many cryopreserved vials are available

for future work. To date, overlay assays have been performed with GSLs extracts from vaginal epithelial cells lines derived from four different individuals, using four representative E. coli isolates. Some assays were performed on cells pre-treated with an inhibitor of GSL synthesis (see Technical Objective 2, Task 3), demonstrating drastic reductions in GSL synthesis demonstrated by bacterial binding in GSL extract from such cell cultures, ranging to complete abolition of binding to some GSL compounds. These data confirm the specificity of the binding assays.

To date, only preliminary assays have been performed with S. saprophyticus, using GSL extracted from bladder and vaginal epithelial cells. Results consistent with our preliminary data were seen but the assays will need further development to obtain publication quality data and images, since our preliminary binding results were obtained with purified GSL standards and other more pure samples. This will be an area of further development for the coming year.

3. Recommendations in relation to the Statement of Work

1. We were pleased to have more GSL samples derived from individual vaginal epithelial cells than anticipated. We are in the process of analyzing the degree of individual variation in binding results in order to assess the appropriateness of pooling samples for our further studies, especially structural characterization. In addition, we will be able to perform immunostaining and bacteria adherence experiments using cells and/or GSL extract from cells derived from the same individual(s). Our results to date suggest that continuing the culture of primary vaginal and bladder epithelial cells throughout the course of the project will be helpful.

2. A major area of development will be optimizing bacterial overlay assays using S. saprophyticus.

d. Task 4, Months 7 to 12: immunostaining assays

1. Experimental methods, assumptions and procedure

One important task we identified at the end of the first year of funding was to develop a non-radioactive method of performing HPTLC immunostaining assays. The overall purpose of these assays is to confirm the identities of GSLs identified through TLC mobilities, using specific MAbs directed against the predicted GSLs. We have previously used the established radioactive assay of Magnani (22), as modified by Kannagi (23) to show that GSL extracts from different cultures of primary bladder epithelial cells contained SGG and DSGG (Stapleton et al., manuscript in preparation), using MAbs RM-1 and ID-4 (both specific for SGG) (21). Last year, we explored two methods of non-radioactive detection of MAb binding to HPTLC plates that showed some promise but were nonetheless rife with frustrations. We have recently developed and refined a new method for detecting MAbs binding GSLs on silica plates using a chemiluminescence substrate with a horse radish peroxidase (HRP) label. This assay is rapid, reproducible, produces strong signals with minimal background, and appears to be highly specific using authentic GSL standards.

In the past few months, we have also been working with Dr. Hakomori to produce fresh lots of the MAbs (TKH-7 and ID4) needed for further development of these immunostaining assays.

Preliminary results using the new assay with authentic GSL standards suggest that these lots contain sufficiently high titers of specific MAb to complete our studies. However, we would like to compare our results with assays done in his laboratory using these MAbs for assays using the original, radioactive methodology in order to externally validate our results

2. Results and discussion

An example of data produced using the newly developed assay and MAb TKH-7, directed against asialo GM-1 is shown in Figure 5. Pieces of glass HPTLC plates were cut and spotted with standard asialo GM1 (Gg4), blocked in BSA/PBS, washed, incubated with a secondary antibody directed against mouse immunoglobulins, washed, and then sequentially incubated with HRP and the chemiluminescence working solution that was provided with the kit. Various dilutions of HRP and secondary antibody were tested as well as varied times of film exposure.

1   2

Figure 5. Binding of MAb TKH-7 to asialo GM1 in an HPTLC immunostaining assay.

An aliquot of standard asialo GM1 was spotted on an HPTLC plate and the plate was subjected to sequential antibody and developing reagent incubations as described above. After final washing, film was exposed for 15 seconds. Two examples of sixteen different combinations of conditions tried in this assay are shown. **Panel 1**: secondary antibody diluted 1:1,000, HRP diluted 1:10,000 (negative); **Panel 2**: secondary antibody diluted 1:10,000, HRP diluted 1:1,000 (positive).

3. Recommendations in relation to the Statement of Work

1. We will expand application of the new non-radioactive assay described above to include samples containing our unknown GSLs of interest and MAb ID4.

2. We will continue to work with Dr. Hakomori to perform the comparative immunostaining studies described above to validate our new method and check the lots of MAb that we will use for the remainder of our studies.

e. Task 5, Months 13 to 24: carbohydrate structural analysis; and

f. Task 6, Months 25 to 36: data analysis and publication

1. Experimental methods, assumptions and procedure

Full details of the methods used in purifying and performing definitive carbohydrate structural analysis of SGG are provided in the appended manuscript (Stroud et al.)

2. Results and discussion

We were pleased to recently receive notice that a manuscript describing the first structural analysis studies funded by this grant (see annual report for Year 1) was accepted for publication in Biochemistry with only minor editorial changes (14). A second manuscript related to this work appeared in Infection and Immunity in August 1998 (4).

3. Recommendations in relation to the Statement of Work

1. In general we are ahead of schedule on these objectives. However, as noted above, we are discussing with Dr. Levery whether any newly available alternative methods for carbohydrate structural analysis are appropriate for our samples. These will be instituted if appropriate.

2. A manuscript describing the data related to S. saprophyticus binding GSLs (see preliminary data) will be submitted shortly and two other manuscripts are in preparation describing data presented in this and last year's report. The methods we use to purify SGG from human primary bladder and vaginal epithelial cells will greatly depend on technical issues related to the methods ultimately used for characterization.

2. Technical Objective 2

a. Task 1, Months 18 to 30: bacterial adherence assays to test representative isolates for adherence to primary bladder cell and vaginal epithelial cultures

1. Experimental methods, assumptions and procedure

We used the same methods described in our preliminary data, as follows: primary cultures of bladder and vaginal epithelial cells were maintained and utilized as described above. E. coli was grown overnight on sheep blood agar plates or on antibiotic-containing Luria agar plates for cloned isolates, harvested in PBS, washed and resuspended to an OD_{600} of 0.5 (corresponding to 5×10^8 organisms). S. saprophyticus organisms were grown overnight shaking in trypticase soy broth and prepared similarly. The organisms were resuspended in 1.0 ml of keratinocyte medium, incubated with the cells for 3 hours, washed repeatedly with PBS and fixed and stained using a commercial Giemsa stain (Baxter). Cells treated with only a change of medium and the nonadherent laboratory isolates HB101 served as controls. For some experiments with the vaginal epithelial cells, we used lines cultivated in parallel form the same individual with and without the induction of morphologically assessed differentiation. In separate preliminary studies recently begun, we adapted a FACS-based adherence assay we routinely use to study bacterial adherence to exfoliated native vaginal

epithelial cells. Cells were grown in suspension and then subjected to our standard bacterial adherence assay conditions. The goal of these studies is to compare data from the living primary cultured vaginal epithelial cells with those obtained with the exfoliated vaginal epithelial cells (unpublished data from our laboratory).

2. Results and discussion

We made a major effort towards end of this funding period to focus our bacterial adherence studies on vaginal epithelial cells since we have some data from the initial year and from our preliminary data on bladder cells. We tested bacterial adherence on seven different vaginal epithelial cells lines, four of which were tested with and without differentiation. Preliminary studies were performed using 7 isolates of E. coli, including four wild type strains and three recombinant strains expressing pap-encoded adhesins. S. saprophyticus ST 352 was used as the representative isolate for studies of S. saprophyticus adherence.

In analyzing the bacterial adherence assays performed on cells grown in chamber slides and using E. coli, we found that adherence of E. coli to undifferentiated cells (morphologies similar to those of basal vaginal epithelial cells in biopsies of normal young women) was generally lower than what we have observed using native exfoliated vaginal epithelial cells, which nearly always have a superficial epithelial cell morphology. When we induced a more differentiated, superficial cell-type morphology in the cultured cells, adherence increased as much as 50 fold. In contrast, adherence of S. saprophyticus ST352 was higher when cells with a basal morphology were used. However, some of our developmental work was done using cells cultured in the characterized keratinocyte medium, which proved detrimental to overall cell health and survival. In part, we chose this medium because its components are simpler than standard keratinocyte medium but a review of our data suggest that adherence was affected as well. In addition, we hypothesize that vaginal epithelial cells likely need exogenous growth factors and/or hormones in order to approach their native state.

3. Recommendations in relation to the Statement of Work

1. We will perform more assays comparing cultured vaginal epithelial cells with or without induction of the superficial morphology, all grown in standard keratinocyte medium. More assays will be preformed using S. saprophyticus ST 352 to confirm our preliminary findings.

2. We will explore the effects on adherence of exposing the vaginal epithelial cell cultures to estrogen and/or progesterone.

3. We will further develop the above-mentioned assay using cells in suspension and FACS-based technology to assess the degree of adherence. These results will be compared with our data from exfoliated vaginal epithelial cells collected from normal young women. In addition, we will explore results of our collaborative study on normal (see above) for possible further directions in defining the state of the cultured vaginal epithelial cells as compared to cells from normal young women.

b. Task 2, Months 18 to 30: immunocytology procedures utilizing immunofluorescence staining of the same cell cultures with MAbs directed against relevant GSLs

1. Experimental methods, assumptions and procedure

During this funding period, we adapted the staining methods reported in our preliminary data for bladder epithelial cells cultured in chamber slides to the vaginal epithelial cell cultures. Our initial goal was to prove more definitively (beyond obvious morphological differences) that our cultures were epithelial cells and not fibroblasts. Thus, our initial studies involved an anti fibroblast surface protein MAb and an anti pan-cytokeratin antibody. MAb NuH2 (15), an unrelated primary antibody, served as our negative control. More recently, we have tested two vaginal epithelial cells lines for staining with MAb ID4.

2. Results and discussion

Figure 6 shows the results of preliminary studies of staining vaginal epithelial cells with MAb ID4, directed against SGG, anti pan-cytokeratin MAb, and NuH2 (15), an unrelated primary antibody.
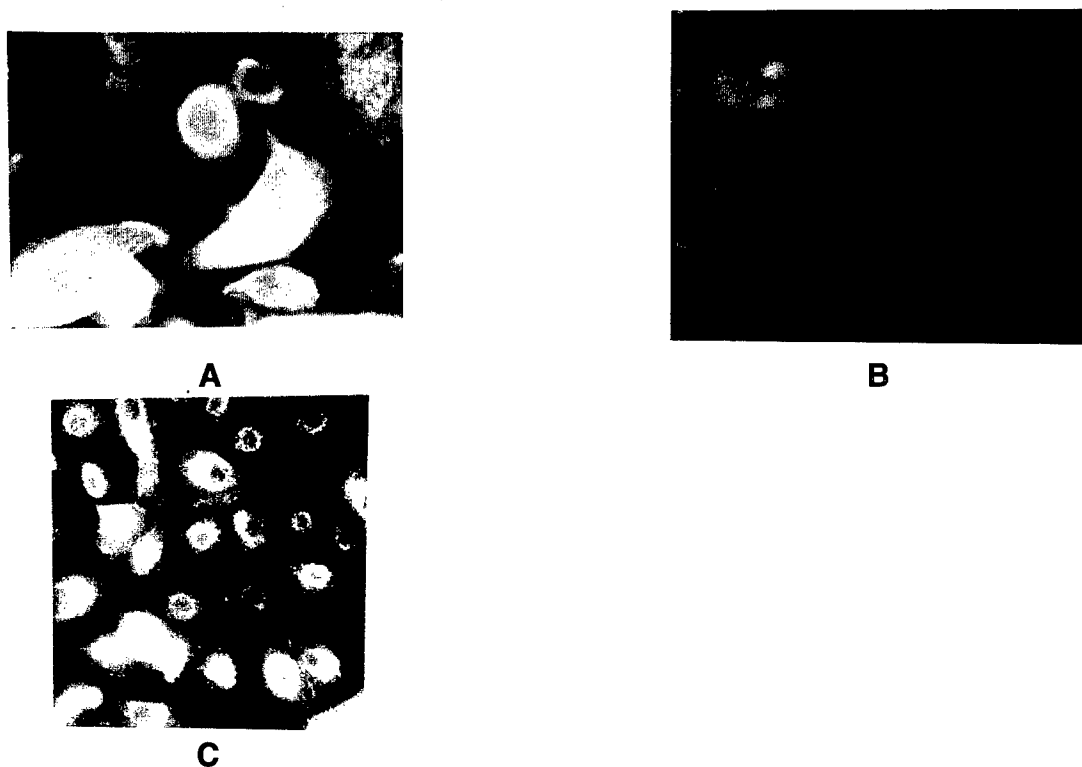
Figure 6. Staining of cultured vaginal epithelial cells with various MAbs.
Cells were fixed briefly and then incubated with the indicated primary MAb, followed by washing and incubation with **(A)** MAb anti pan cytokeratin (positive control; 40X); **(B)** NuH2 (unrelated primary MAb; negative control; 40X); and **(C)** MAb ID4, directed against SGG (20X). Cells were then incubated with a FITC-conjugated secondary antibody directed against mouse IgG, washed and photographed.

3. Recommendations in relation to the Statement of Work

These studies will be greatly expanded in the next funding year, especially since they were begun when we had not yet standardized culture conditions for primary vaginal epithelial cells.

c. Task 3, Months 24 to 36: PDMP treatment of cell cultures, followed by GSL extraction and quantification and bacterial adherence assays

1. Experimental methods, assumptions and procedure

Several inhibitors of GSL synthesis, such as 1-phenyl-2-(decanoylamino)-3-morpholino-1-propanol (PDMP), an analog of glucosylceramide that competitively inhibits the synthesis of GSLs in living cells, have been synthesized (27) and some are available on the market. We performed pilot experiments to show that specific GSL synthesis inhibition was important in bacterial adherence to bladder epithelial cells by concomitantly performing bacterial adherence assays and extracting and quantifying the GSLs in treated versus untreated cultures. For this year of funding, we began using a related compound, 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPPP) because it is the most active congener of this group of compounds but has effects similar to those of PDMP in tissues where it has been studied. We performed preliminary experiments with this compound using techniques described in our preliminary data. Briefly, vaginal epithelia cell cultures were treated with PPPP and then one of the following assays were performed, as described above: (1) extraction of GSLs form cell pellets; (2) bacterial overlay assays using these cell pellets; and (3) bacterial adherence assays using intact, pre-treated cells.

2. Results and discussion

As shown in Figures 1 and 4, PPPP drastically reduces or abolished GSL synthesis in the vaginal epithelia cell cultures system, as evidenced by reduced or absent staining with orcinol and near abolition of bacterial binding using E. coli as a probe. In addition, in preliminary experiments we have recently begun, PPPP treatment of intact cell cultures reduced the adherence of E. coli R45 from approximately 50 bacteria/cell to 0 bacteria/cell.

3. Recommendations in relation to the Statement of Work

Since these investigations appear successful, we plan no changes in this protocol at present. They will be expanded over the next year to repeat and confirm preliminary data, including performing additional bacterial adherence assays with both E. coli and S. saprophyticus as well as bacterial overlay assays with S. saprophyticus.

3. Technical Objective 3

a. Task 1, Months 24 to 42: synthesis of linear mimetic compounds

As noted above, we met with Dr. Toyokuni in August at the XIX International Carbohydrate Symposium to plan how to begin the synthesis of the mimetic compounds. Dr. Toyokuni is now a faculty member in the Department of Chemistry at UCLA and has access to a broader range of equipment, reagents, and consulting assistance than he did when this proposal was first funded. However, we discussed a few logistical changes in our approach to this technical objective, such as beginning by modifying the existing compound of interest (SGG). To this end, in the past year, Dr. Stroud has begun pursuing two new, separate methods of generating relatively large quantities of SGG for these chemical modification studies. Our first approach is based on an early report on the presence of SGG in chicken pectoral muscle (16). The availability and low cost of this tissue makes it an ideal source for the purification of large amounts of SGG. Our second approach is based on the partial enzymatic synthesis of galactosyl globoside (GG), the immediate precursor to SGG. We have detected relatively high levels of ß1-3 galactosyltransferase in two separate cell lines capable of catalyzing the biosynthesis of GG using globoside as an acceptor substrate.

2. Results and discussion

We began working with the chicken muscle tissue several months ago, starting with about 800 g of tissue. We have detected a glycolipid that co-migrates with SGG in ganglioside fractions of chicken muscle and binds E. coli using our HPTLC bacterial overlay assay, as shown below in Figure 7:
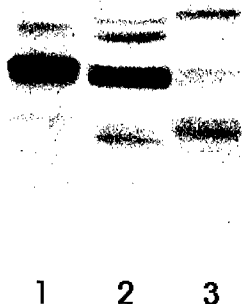
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1     2     3
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Figure 7. Binding of E. coli R45 to mono- and disialylated GSL fractions of chicken pectoral muscle GSLs

Chicken pectoral muscle tissue was ground, subjected to sequential organic extraction in isopropanol:hexane:water (55:25:20 vol:vol), reverse phase column chromatography, a modified Folch partition procedure, and anion exchange column chromatography. Samples of the mono- and disialylated fractions were separated on HPTLC and plates were then overlaid with metabolically [^{35}S]methionine-labeled E. coli R45. **Lane 1**: SGG standard from human kidney; **Lane 2**: monosialyl fraction; **Lane 3**: disialyl fraction.

This material has been purified to the point of being ready for HPLC purification of specific bands of interest. Our initial extraction of this quantity of tissue should supply sufficient amounts of SGG for structural confirmation.

3. Recommendations in relation to the Statement of Work

1. We will purify the compound(s) co-migrating with standard SGG isolated from chicken muscle shown above, verify its carbohydrate and lipid structure in collaboration with Dr. Levery, and then send it to Dr. Toyokuni for chemical modification studies aimed at determining which chemical groups are most important for bacterial adherence. Further preparations of SGG from chicken muscle will be performed as needed.

2. We will concomitantly pursue the enzymatic approach to producing larger quantities of SGG. Once sufficient amounts of GG are synthesized we will sialylate the terminal galactose of GG using CMP-sialic acid as the donor substrate and a commercially available α2,3 sialyltransferase.

D. Publications during the second year of funding

1.   Johnson JR, Russo TA, Brown JJ, Stapleton A. papG alleles of Escherichia coli strains causing first-episode or recurrent acute cystitis in adult women. J Infect Dis 1998;177:97-101.

2.   Gupta K, Stapleton AE, Hooton TM, Roberts PL, Fennell CL, Stamm WE. Inverse association of H_2O_2-producing Lactobacilli and vaginal Escherichia coli colonization in women with recurrent urinary tract infection. J Infect Dis 1998; 178:446-450.

3.   Island MD, Cui X, Foxman B, Marrs CF, Stamm WE, Stapleton AE, Warren JW. Cytotoxicity of hemolytic, cytotoxic necrotizing factor 1-positive and -negative Escherichia coli towards human T24 bladder cells. Infect Immun 1998;66:3384-3389.

4.   Johnson DE, Lockatell CV, Russell RG, Hebel JR, Island MD, Stapleton A, Stamm WE, Warren JW. Comparison of Escherichia coli recovered from human cystitis and pyelonephritis infections in transurethrally challenged mice. Infect Immun 1998;66:3059-3065.

5.   Stapleton AE, Stroud MR, Hakomori SI, Stamm WE. The globo-series glycosphingolipid sialosyl galactosyl globoside is found in urinary tract tissues and is a preferred binding receptor in vitro for uropathogenic Escherichia coli expressing pap-encoded adhesins. Infect Immun 1998;66:3856-3861.

6.   Ummenhofer W, Stapleton A, Bernards C. Effect of Staphylococcus aureus bacteria and bacterial toxins on meningeal permeability in vitro. Reg Anesth and Pain Med 1998;in press.

7.   Stroud MR, Stapleton AE, Levery SB. The P-blood group related glycosphingolipid sialosylgalactosylgloboside (SGG) as a preferred ligand for uropathogenic E. coli: Isolation and structural characterization of SGG from human kidney. Biochemistry 1998; in press.

7. CONCLUSIONS

1. In the first year of funding, our studies were largely aimed at demonstrating the feasibility of our approach of using pure, primary cultures of human bladder and vaginal epithelial cells to identify, purify and structurally characterize E. coli- and S. saprophyticus-binding glycosphingolipid moieties. Vaginal epithelial cell cultures were established de novo but the techniques we employed were not optimized. In the second year of funding, we have refined the techniques we developed for culturing primary vaginal epithelial cells and greatly expanded the scope of this work, developing a unique model for the study of a tissue that is critically important in the bacterial colonization stages that precede the development of bladder inoculation and UTI. Key aspects of this technique that have been optimized include minimizing time to attachment and eliminating fibroblast contamination.

2. Stocks of cryopreserved vaginal epithelial cells have been greatly expanded and we have established ongoing collaborations that will continue to provide fresh material for the establishment of new cell lines for the remainder of the funding of this project, should this be necessary.

3. We have substantially accelerated the pace of GSL purification from both bladder and vaginal epithelial cell lines, but particularly from the latter. Our collaboration with Dr. Steven Levery of the Complex Carbohydrate Research Center, initiated in the first funding period, came to fruition with the publication of our manuscript (see appendix). This collaboration will continue during the next funding period, during which time we will explore new methods of carbohydrate structural determination available to Dr. Levery that require very small amounts of material.

4. We have shown that GSLs from primary vaginal epithelial cells from various individuals contain extended globoseries GSLs, including a structure co-migrating with standard SGG.

5. We have published two manuscripts directly related to this work and have published 5 other manuscripts that are in the same general sphere of investigation.

6. We have confirmed our preliminary finding that primary vaginal epithelial cells serve as a useful model for studying the adherence of uropathogenic bacteria in the pathogenesis of UTI.

7. We have developed a new, non-radioactive method for immunostaining GSLs on HPTLC plates.

8. We have extended our studies of inhibitors of GSL synthesis (PDMP and PPPP) demonstrate that GSLs are also critically important in the adherence of uropathogenic E. coli to vaginal epithelium.

9. We have developed two new approaches for preparing SGG from alternative sources other than precious urinary tract tissues.

8. REFERENCES

1.   Mabeck CE. Treatment of uncomplicated urinary tract infection in nonpregnant women. Postgrad Med J 1972;48:69-75.

2.   Stamm WE, McKevitt M, Roberts PL, White NJ. Natural history of recurrent urinary tract infections in women. Rev Infect Dis 1991;13:77-84.

3.   Stapleton A, Nudelman E, Clausen H, Hakomori S-I, Stamm WE. Binding of uropathogenic Escherichia coli R45 to glycolipids extracted from vaginal epithelial cells is dependent on the histo-blood group secretor status. J Clin Invest 1992;90:965-972.

4.   Stapleton AE, Stroud MR, Hakomori SI, Stamm WE. The globo-series glycosphingolipid sialosyl galactosyl globoside is found in urinary tract tissues and is a preferred binding receptor in vitro for uropathogenic Escherichia coli expressing pap-encoded adhesins. Infect Immun 1998;66:3856-3861.

5.   Kinane DF, Blackwell CC, Brettle RP, Weir DM, Winstanley FP, Elton RA. ABO blood group, secretor state and susceptibility to recurrent urinary tract infection in women. Br Med J 1982;28:7-9.

6.   Karlsson K-A. Animal glycosphingolipids as membrane attachment sites for bacteria. Annu Rev Biochem 1989;58:309-350.

7.   Krivan HC, Roberts DD, Ginsburg V. Many pulmonary bacteria bind specifically to the carbohydrate sequence GalNAcβ1-4Gal found in some glycolipids. Proc Natl Acad Sci USA 1988;85:6157-6161.

8.   Boren T, Falk P, Roth KA, Larson G, Normark S. Attachment of Helicobacter pylori to human gastric epithelium mediated by blood group antigens. Science 1993;262:1892-1895.

9.   Harouse JM, Bhat S, Spitalnik SL, et al. Inhibition of entry of HIV-1 in neural cell lines by antibodies against galactosyl ceramide. Science 1991;253:320-323.

10.   Brown KE, Anderson SM, Young NS. Erythrocyte P antigen: cellular receptor for B19 parvovirus. Science 1993;262:114-117.

11.   Hakomori S-I. Glycosphingolipids in cellular interaction, differentiation, and oncogenesis. Annu Rev Biochem 1981;50:733-764.

12.   Johnson JR. Virulence factors in Escherichia coli urinary tract infection. Clin Microbiol Rev 1991;4:80-128.

13.   Cilento BG, Freeman MR, Shneck FX, Retik AB, Atala A. Phenotypic and cytogenetic characterization of human bladder urothelia expanded in vitro. J Urol 1994;152:665-670.

14.   Stroud MR, Stapleton AE, Levery SB. The P-blood group related glycosphingolipid sialosylgalactosylgloboside (SGG) as a preferred ligand for uropathogenic E. coli: Isolation and
structural characterization of SGG from human kidney. Submitted 1998.

15.   Nudelman ED, Mandel U, Levery SB, Kaizu T, Hakomori S. A series of disialogangliosides with binary 2-3 sialosyllactosamine structure, defined by monoclonal antibody NUH2, are oncodevelopmentally regulated antigens. J Biol Chem 1989;264:18719-18725.

16.   Chien J-W, Hogan EL. Novel pentahexosyl ganglioside of the globo series purified from chicken muscle. J Biol Chem 1983;258:10727-10730.

9. APPENDICES (attached)

Appendix A: reprint of Stapleton et al., Infect Immun 1998;66:3856-3861.

Appendix B: pre-print of Stroud et al., Biochemistry 1998:in press.

The Globoseries Glycosphingolipid Sialosyl Galactosyl Globoside Is Found in Urinary Tract Tissues and Is a Preferred Binding Receptor In Vitro for Uropathogenic *Escherichia coli* Expressing *pap*-Encoded Adhesins

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Women with a history of recurrent *Escherichia coli* urinary tract infections (UTIs) are significantly more likely to be nonsecretors of blood group antigens than are women without such a history, and vaginal epithelial cells (VEC) from women who are nonsecretors show enhanced adherence of uropathogenic *E. coli* isolates compared with cells from secretors. We previously extracted glycosphingolipids (GSLs) from native VEC and determined that nonsecretors (but not secretors) selectively express two extended globoseries GSLs, sialosyl galactosyl globoside (SGG) and disialosyl galactosyl globoside (DSGG), which specifically bound uropathogenic *E. coli* R45 expressing a P adhesin. In this study, we demonstrated, by purifying the compounds from this source, that SGG and DSGG are expressed in human kidney tissue. We also demonstrated that SGG and DSGG isolated from human kidneys bind uropathogenic *E. coli* isolates expressing each of the three classes of *pap*-encoded adhesins, including cloned isolates expressing PapG from J96, PrsG from J96, and PapG from IA2, and the wild-type isolates IA2 and R45. We metabolically ^{35}S labeled these five *E. coli* isolates and measured their relative binding affinities to serial dilutions of SGG and DSGG as well as to globotriaosylceramide (Gb$_3$) and globotetraosylceramide (Gb$_4$), two other globoseries GSLs present in urogenital tissues. Each of the five *E. coli* isolates bound to SGG with the highest apparent avidity compared with their binding to DSGG, Gb$_3$, and Gb$_4$, and each isolate had a unique pattern of GSL binding affinity. These studies further suggest that SGG likely plays an important role in the pathogenesis of UTI and that its presence may account for the increased binding of *E. coli* to uroepithelial cells from nonsecretors and for the increased susceptibility of nonsecretors to recurrent UTI.

Several epidemiological studies have shown that women who are nonsecretors of blood group antigens have a three- to fourfold-increased risk of developing recurrent urinary tract infection (UTI) (5, 17, 32). In addition, uroepithelial cells from nonsecretors have a two- to threefold-greater capacity for adherence of uropathogenic *Escherichia coli* than do cells from secretors (22). Colonization of the vaginal and periurethral epithelium precedes the development of *E. coli* UTI, and *E. coli* isolates expressing *pap*-encoded adhesins are overrepresented among isolates causing these infections (6). Uropathogenic *E. coli* isolates expressing *pap*-encoded adhesins bind to globoseries glycosphingolipids (GSLs) (6, 19), which are amphipathic molecules embedded in the outer leaflets of eukaryotic cell membranes. There are several families of GSLs which are differentiated by their molecular structures, and these molecules serve as bacterial and viral adhesion sites on mammalian cells and as markers of eukaryotic cell differentiation and oncogenesis (4).

In previous investigations, we collected vaginal epithelial cells from secretors and nonsecretors and extracted the GSLs from pooled cells from women in each group (36). We demonstrated that cells from nonsecretors express two unique globoseries GSLs, sialosyl galactosyl globoside (SGG) and disialosyl galactosyl globoside (DSGG) (36). We utilized high-performance thin-layer chromatography (HPTLC), bacterial overlay assays, HPTLC immunostaining, radioimmunoassays, and immunohistochemical staining with a monoclonal antibody (MAb) directed against SGG to show that SGG and DSGG were expressed in vaginal epithelial cells from nonsecretors but not in cells from secretors and that these moieties bound a clinical isolate of *E. coli* (R45) that expresses P fimbriae carrying a *pap*-encoded adhesin (36). These studies demonstrated for the first time that the secretor gene influences the biosynthesis of globoseries GSLs in the vaginal epithelium and suggested that genetically determined differences in receptor moieties in this tissue might explain the increased susceptibility of nonsecretors to UTI (32, 36).

In this study, we isolated and purified SGG and DSGG from human kidneys and assessed the in vitro binding of representative Pap-expressing *E. coli* isolates to SGG and DSGG in order to further elucidate possible mechanisms through which the selective expression of one or both of these molecules in the vaginal or urogenital epithelium of nonsecretors might influence their risk of UTI.

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TABLE 1. Structures of GSL standards used in this study

GSL[a]	Structure[b]	Source
CMH	Glcβ1-1cer	Colonic adenocarcinoma
CTH	Galα1-4 Galβ1-4 Glcβ1-1cer	Human erythrocytes
Globoside	GalNAcβ1-3 Galα1-4 Galβ1-4 Glcβ1-1cer	Human erythrocytes
Gal globoside	Galβ1-3 GalNAcβ1-3 Galα1-4 Galβ1-4 Glcβ1-cer	Human kidney
SGG	NeuAcα2-3 Galβ1-3 GalNAcβ1-3 Galα1-4 Galβ1-4 Glcβ1-1cer	Human kidney
DSGG	NeuAcα2-3 Galβ1-3 (NeuAcα2-6) GalNAcβ1-3 Galα1-4 Galβ1-4 Glcβ1-1cer	Human kidney
Forssman	GalNAcα1-3 GalNAcβ1-3 Galα1-4 Galβ1-4 Glcβ1-1cer	Goat erythrocytes
ASGM1	Galβ1-3 GalNAcβ1-4 Galβ1-4 Glcβ1-1cer	Desialylated GM1 from bovine brain

[a] Globoside, Gb₄ (globotetraosylceramide); Gal globoside, galactosyl globoside; ASGM1, asialo-GM1.

[b] Glc, glucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; NeuAc, *N*-acetylneuraminic (sialic) acid; cer, ceramide.

MATERIALS AND METHODS

Purification of SGG and DSGG from human kidney tissue. Normal human kidney tissue was chosen as an appropriate source from which to purify SGG and DSGG for several reasons. First, it is an available and clinically relevant urinary tract tissue, whereas the vaginal epithelium cannot be harvested in sufficient quantity for the purification of SGG and DSGG. In addition, we chose a human tissue as the source for these compounds, since variations in the structure of the ceramide portions of GSLs may be species specific, and thus structural differences found in animal tissues can have implications for the binding specificities of microorganisms (14). In preliminary studies, using the methods described below, we extracted and purified GSLs from small autopsy samples of normal human kidney tissue and determined that SGG and DSGG were expressed in these tissues. The purification was then scaled up, and a total of 1 kg of normal human kidney tissue was obtained and pooled from autopsy specimens from eight individuals. Autopsy reports were reviewed to insure that none of the patients died from renal disease or from diseases affecting kidney function. The majority of the material by weight was obtained from a 38-year-old woman who died from medulloblastoma. The tissue was washed and homogenized in a Waring blender, and GSLs were then prepared by a series of standard purification steps. First, an organic extraction with isopropanol-hexane-water was performed (10), followed by a modified Folch extraction (3) to produce lower and upper phases. No further purification of the lower phase was performed for these studies. The upper phase was then subjected to anion-exchange chromatography (41). Neutral GSL fractions were collected in the flowthrough, and acidic fractions were eluted with 0.05, 0.15, and 0.45 M ammonium acetate washes. The neutral fraction was then further purified by reverse-phase chromatography followed by acetylation and deacetylation to remove phospholipids and cholesterol (40, 41). The acidic fractions were then subjected to normal-phase silica gel high-performance liquid chromatography (HPLC) (13). SGG and DSGG were then identified and purified from the HPLC fractions by stepwise combinations of HPTLC immunostaining (12, 24), bacterial overlay assays (36), HPTLC in multiple solvent systems, and preparative HPTLC (28). The purification of SGG and DSGG as well as the structural characterization of SGG will be described more fully elsewhere (37a).

HPTLC immunostaining and bacterial overlay assays. GSLs isolated and purified from the kidney tissues and then separated on HPTLC were immunostained according to the procedure of Magnani et al. (24), as modified by Kannagi et al. (12). Briefly, after HPTLC, the plates were blocked for 2 h in 5% bovine serum albumin in phosphate-buffered saline, washed, and incubated with the primary MAb in phosphate-buffered saline. After an incubation with the secondary antibody, the plates were washed, incubated with ^{125}I-labeled protein A solution, washed, dried, and subjected to autoradiography. MAbs ID4 and RM-1, directed against SGG (31, 36), were used to monitor the purification of both SGG and DSGG. Since DSGG differs in structure from SGG by only one sialic acid residue, DSGG was identified by subjecting the compound to a timed, limited desialylation procedure to produce SGG (27). Briefly, aliquots of the purified putative DSGG compound to be tested were incubated in 1% acetic acid for 1, 3, and 7 min at 100°C and the reactions were terminated by plunging the tubes in ice and adding ice-cold ethanol. The samples were then dried and subjected to HPTLC, and immunostaining with MAb ID4 was performed. The presence of globoseries GSL moieties, particularly SGG and DSGG, was also monitored in the various fractions obtained during the lengthy purification steps with HPTLC bacterial overlay assays. Assays were performed as previously described (36) with metabolically ^{35}S-labeled *E. coli* isolate R45, a wild-type cystitis isolate (35) which expresses P fimbriae carrying the class II *pap*-encoded adhesin (9) and binds globoseries GSLs (36). At every step, the results of HPTLC immunostaining and bacterial overlay experiments were compared, and relevant fractions and individual bands visualized by HPTLC were then subjected to further purification, as described above.

Bacterial binding curves. (i) GSL standards. Globoseries GSL standards were isolated and purified in our laboratory from the following sources, using methods similar to those described above for purifying SGG from human kidney tissue (29): (i) ceramide trihexoside (CTH; globotriaosylceramide [Gb₃]), from human

erythrocytes; (ii) globoside (globotetraosylceramide [Gb₄]), from human erythrocytes; and (iii) SGG and DSGG, purified from human kidney tissue as described above. Ceramide monohexoside (CMH) was purified from colonic adenocarcinoma and was used as a negative control for the binding of *E. coli* expressing P fimbriae carrying *pap*-encoded adhesins (36, 37). GSL standards were quantitated by a combination of the resorcinol and sphingosine assays (25) and densitometry. Relative quantities of GSLs were standardized by HPTLC by the comparative dilution method, using an appropriate reference GSL having a carbohydrate chain of equal length and charge and of similar molarity to that of the GSL being standardized. (34). The structures of the compounds utilized are shown in Table 1.

(ii) Bacterial binding assays. To construct binding curves, GSL standards were serially diluted on HPTLC plates from 300 to 18.25 ng and overlaid with metabolically ^{35}S-labeled *E. coli* isolates in bacterial overlay assays, as previously described (36). This range of GSL concentrations was chosen on the basis of preliminary experiments with two of the *E. coli* isolates described below that showed saturation of bacterial binding for SGG at higher concentration ranges of these GSL standards. After bacterial overlay, the HPTLC plates were subjected to autoradiography, and densitometry of the autoradiographs was performed to assess the quantity of bacterial binding to each GSL relative to the others. A second method of assessing bacterial binding, using the same plates, was performed by scraping the silica band corresponding to bacterial binding to each GSL standard, followed by counting the radioactivity in a scintillation counter.

***E. coli* isolates.** The *E. coli* isolates that were tested included the following: (i) R45, a wild-type cystitis isolate from a woman with acute cystitis which expresses P fimbriae carrying a class II *pap*-encoded adhesin (8, 35); (ii) IA2, a second wild-type isolate, from which HB101/pDC1 (called pDC1 in this paper) was cloned and which expresses P fimbriae carrying a class II *pap*-encoded adhesin (2); (iii) JJ122, which expresses P fimbriae carrying a class I *papG*-encoded adhesin (PapG from J96) (HB101/pJJ48); (iv) pDC1, which expresses P fimbriae carrying a class II *pap*-encoded adhesin (PapG from IA2) (2); (v) P678-54/pJFK102 (called pJFK102 in this paper), which expresses P fimbriae carrying a class III adhesin (PrsG from J96) (15); and (vi) the negative control isolates HB101 and P678-54. Wild-type organisms were grown on sheep's blood agar, and recombinant isolates were grown on Luria broth agar plates containing the appropriate antibiotics. Under the growth conditions utilized for these studies, type 1 fimbriae were not expressed by any of the isolates (data not shown).

RESULTS

Purification of SGG and DSGG from human kidney tissue. As described above, the purification of SGG and DSGG was monitored by HPTLC immunostaining and bacterial overlay assays on fractions putatively containing the compounds of interest. The results of performing HPTLC immunostaining on samples of purified SGG and DSGG, using MAb RM-1 directed against SGG (31), are shown in Fig. 1. The MAb stained only the band corresponding to SGG and did not stain DSGG or the negative control GSL, ceramide trihexoside (Gb₃). The results of experiments to identify DSGG are shown in Fig. 2. In these experiments, the fraction putatively containing DSGG was subjected to a timed, limited desialylation procedure to produce SGG, followed by HPTLC and immunostaining with MAb ID4, directed against SGG (36). A comparison of lanes 1 to 3 in Fig. 2 shows that increasing amounts of SGG are produced over time through desialylation of DSGG, resulting in increasing staining of a band corresponding to SGG on the autoradiograph of MAb ID4 staining shown in Fig. 2A. In the
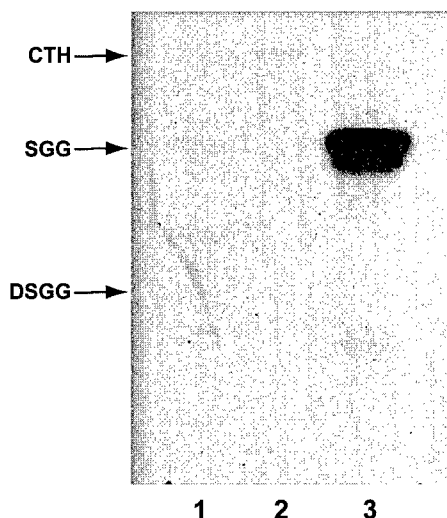
FIG. 1. Identification of SGG purified from human kidney tissue by HPTLC immunostaining. GSL standards, including SGG and DSGG purified from human kidney tissue, were chromatographed and immunostained with MAb RM-1, directed against SGG, as described in Materials and Methods. Lane 1, ceramide trihexoside (Gb3; negative control); lane 2, DSGG; lane 3, SGG.

FIG. 3. Binding of representative E. coli isolates expressing pap-encoded adhesins to SGG and DSGG purified from human kidney tissue. GSLs were purified and separated on HPTLC plates and then overlaid with metabolically [^{35}S]methionine-labeled E. coli R45 organisms, which bind globoseries GSLs. Autoradiographs are shown. (A) E. coli R45; GSLs: ganglioside fraction (lane 1), DSGG standard (lane 2), DSGG standard isolated from a different preparation and subjected to formal carbohydrate structural analysis (31) (lane 3), SGG standard (note traces of DSGG and galactosyl globoside) (lane 4), and CTH (Gb$_3$) and Forssman (Forss) standards (lane 5). (B) E. coli JJ122; GSLs DSGG standard (lane 1), blank (no GSLs) (lane 2), SGG standard (lane 3), asialo-GM1 (ASGM1) standard (lane 4), and CTH and Forssman standards (lane 5). (C) E. coli pJFK102; GSLs: DSGG standard (lane 1), DSGG standard isolated from a different preparation and subjected to formal carbohydrate structural analysis (lane 2), SGG (lane 3), ASGM1 standard (lane 4), and CTH and Forssman standards (lane 5).

replicate HPTLC plate stained with orcinol (Fig. 2B), this is reflected by a reduction in orcinol staining of the band corresponding to DSGG, along with an increase in staining of the band corresponding to SGG. At the 7-min desialylation time point, a portion of the sample has likely also been converted to galactosyl globoside, seen as a faint band migrating more rapidly than SGG in lane 3.
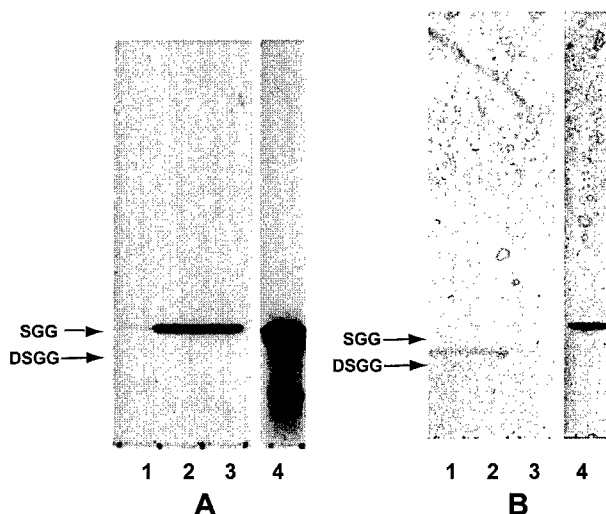

FIG. 2. Identification of DSGG purified from human kidney tissue by timed, limited desialylation of DSGG to SGG, followed by HPTLC immunostaining. DSGG purified from human kidney tissue was identified through desialylation to form SGG, followed by immunostaining. A putative DSGG fraction was subjected to a limited desialylation procedure by incubating aliquots of the sample for 1, 3, and 7 min in 1% acetic acid and then drying the samples, subjecting them to HPTLC, and performing TLC immunostaining with MAb ID4, directed against SGG. (A) Autoradiograph of immunostained HPTLC plate; (B) same HPTLC plate stained with orcinol reagent after the immunostaining procedure. Lanes 1, DSGG fraction after 1 min of desialylation of DSGG; lanes 2, DSGG fraction after 3 min of desialylation; lanes 3, DSGG fraction after 7 min of desialylation; lanes 4, SGG standard.
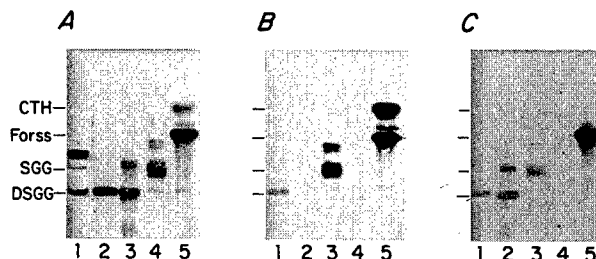
Figure 3 shows an example of the multiple bacterial overlay experiments used to purify SGG and DSGG as well as the final result of these purification steps. These experiments demonstrate that SGG and DSGG purified from human kidney tissue bind metabolically ^{35}S-labeled representative E. coli isolates R45, JJ122, and pJFK102, expressing P fimbriae carrying pap-encoded adhesins of classes II, I, and III, respectively. Nineteen bacterial overlay experiments were performed during the course of purifying SGG and DSGG (12 experiments using E. coli R45 and 7 experiments using one or more of the other four E. coli isolates described above). We repeatedly observed qualitative differences between the avidities of bacterial binding to SGG and to CTH and other GSL standards with shorter-chain oligosaccharide moieties, based on comparing orcinol staining of known quantities of these purified GSL standards with the amount of SGG in samples still in the process of purification. For example, bacterial binding to 5 to 10 μg of CTH GSL standard was approximately equivalent to the amount of binding to SGG that was at the limits of staining with orcinol, estimated at 20 ng or less. These observations led to the bacterial binding quantitation experiments described below, using purified SGG and DSGG.

Bacterial binding curves. The results of quantitating bacterial binding to serially diluted CTH, globoside, SGG, and DSGG standards both by means of scraping and counting radioactive bands from the silica plates and by performing densitometry of the autoradiographs were essentially identical. Figure 4 shows the autoradiographs from these experiments (left panels) as well as the results of counting radioactivity scraped from bands on silica plates corresponding to binding of metabolically ^{35}S-labeled E. coli isolates R45, IA2, pDC1, JJ122, and pJFK102 (right panels). Results of representative experiments are shown for each strain. For each isolate, the relative binding to SGG was greater than the binding to other globoseries GSLs tested. No binding of GSLs by HB101 or in bacterial overlay assays was observed, even when the plates were exposed to film for 7 days (data not shown).

DISCUSSION

In a previous study, we demonstrated that vaginal epithelial cells from nonsecretors selectively express SGG and DSGG
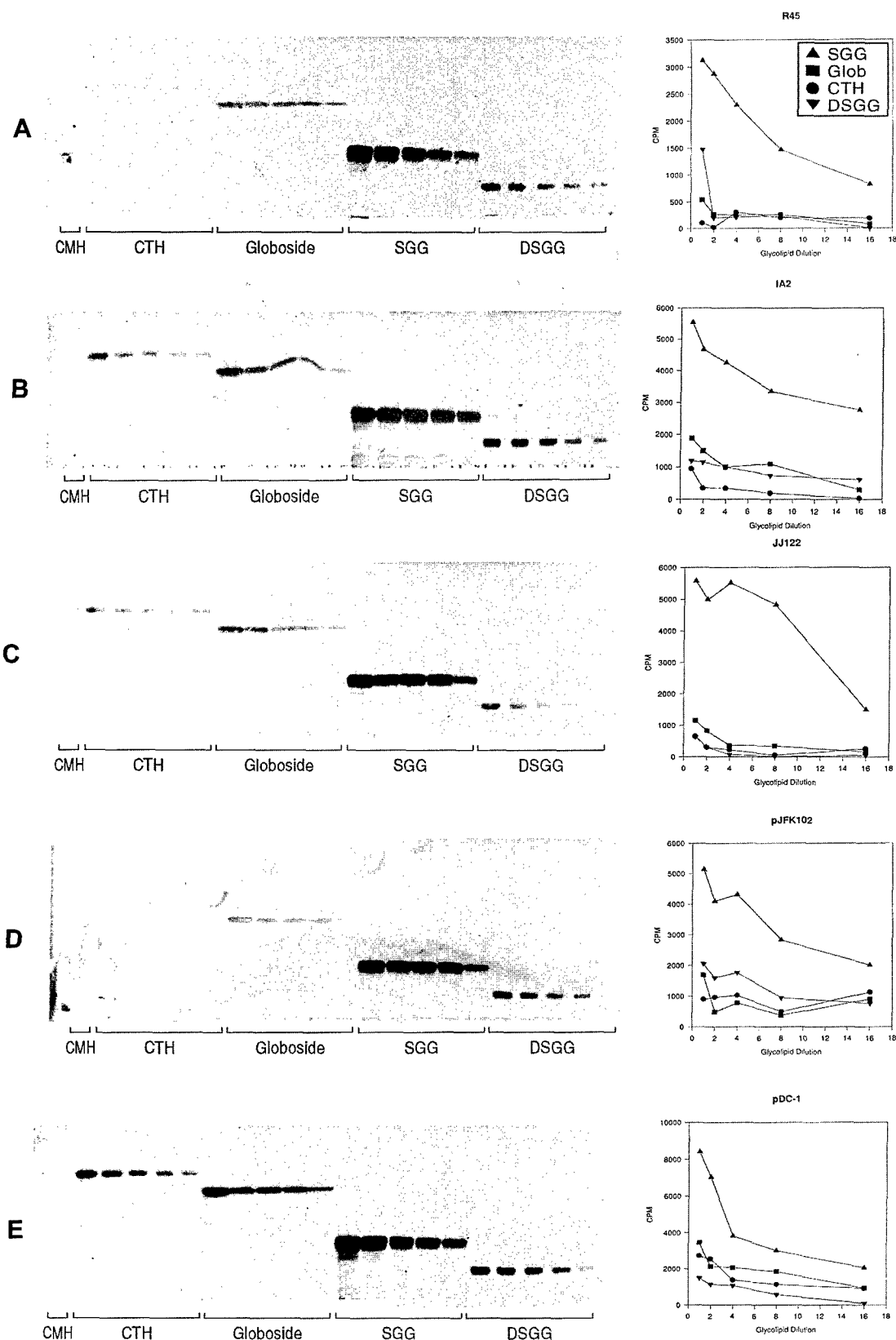
FIG. 4. Binding of representative *E. coli* isolates to serial dilutions of globoseries GSLs in bacterial overlay assays. Autoradiographs of binding are shown in the lefthand panels, and the righthand panels show the quantification of the binding demonstrated in the adjoining autoradiographs. GSLs were serially diluted from 300 to 18.25 ng, chromatographed by HPTLC, and overlaid with representative *E. coli* isolates, as described in the text. The lanes containing standards for CMH (5 μg; negative control), CTH (Gb3), globoside, SGG, and DSGG are labeled. (A) *E. coli* R45; (B) *E. coli* IA2; (C) *E. coli* JJ122; (D) *E. coli* pJFK102; (E) *E. coli* pDC1. Glob, globoside.

and that these compounds bind a wild-type uropathogenic *E. coli* strain, R45, expressing a *pap*-encoded adhesin (36). Binding did not occur under conditions where *pap*-encoded adhesins were not expressed (36). We reasoned that the presence of these *E. coli*-binding GSLs on the vaginal epithelial cells of nonsecretors but not secretors might explain the increased propensity of nonsecretors for developing recurrent UTIs (5, 17, 32). In the studies reported here, we have now shown that SGG and DSGG are also expressed in human kidney tissues and that these compounds, purified from this source, bind cloned and wild-type uropathogenic *E. coli* isolates expressing *pap*-encoded adhesins. These strains represent the three known classes of P fimbrial adhesins. Using a PCR method that distinguishes the three classes of adhesins (7), we previously determined that *E. coli* R45 expresses P fimbriae carrying a class II adhesin (8, 9). In addition, we demonstrated the binding of SGG and DSGG by IA2, another wild-type isolate expressing P fimbriae carrying a class II *pap*-encoded adhesin, as well as by a cloned isolate expressing this adhesin (PapG from IA2), pDC1 (2). The class I *papG*-encoded adhesin was represented by an isolate expressing P fimbriae carrying PapG from J96 (HB101/pJJ48), expressing the *pap* operon from pHU845 (26), and the class III *papG*-encoded adhesin was represented by pJFK102, which expresses P fimbriae carrying PrsG from J96 (15). Thus, we have demonstrated that SGG and DSGG are relevant bacterial binding moieties for uropathogenic *E. coli* isolates expressing P fimbriae carrying all three known members of the family of *pap*-encoded adhesins.

To investigate the possible biological implications of this finding, we designed experiments to assess the relative binding of these *E. coli* isolates to the GSLs SGG and DSGG (nonsecretor-restricted in the vaginal epithelium [36]) as well as to other relevant globoseries GSLs that we previously identified on both secretors' and nonsecretors' vaginal epithelial cells (36). Before the various classes of *pap*-encoded adhesins were genetically defined, the binding of various wild-type and cloned uropathogenic *E. coli* isolates expressing *pap*-encoded adhesins to globoseries GSLs other than SGG and DSGG was investigated (21, 37). These studies demonstrated relatively little difference between GSL binding to globoside and binding to Gb_3 for those *E. coli* isolates expressing P fimbriae carrying *pap*-encoded adhesins of classes I or II. Isolates expressing P fimbriae carrying a class III *pap*-encoded adhesin demonstrated a preference for binding to extended globoseries GSLs (37). In preliminary experiments, we found that binding of *E. coli* to SGG and DSGG was completely saturated in the GSL concentration range (0 to 1.0 μg) reported in one of these two previous studies, in which a similar technique was used (21, 37). Thus, we constructed GSL binding curves in lower concentration ranges (18 to 300 ng). Although we confirmed most of the previously reported data regarding the relative efficiency of binding of *E. coli* expressing P fimbriae carrying *pap*-encoded adhesin(s) to globoseries GSLs such as Gb_3 and Gb_4, we found that all five *E. coli* isolates bound more strongly to SGG than to the other globoseries GSLs tested, including DSGG. These data demonstrate that, at least in the urogenital epithelia of nonsecretors, SGG may be a preferred ligand for uropathogenic *E. coli* isolates.

In the studies reported here, we have isolated and purified SGG and DSGG from normal human kidney tissue for the first time. Further structural analysis of the SGG sample we obtained from this tissue source by proton nuclear magnetic resonance spectroscopy, mass spectroscopy, and linkage analysis has been completed and will be reported elsewhere (37a), while similar chemical characterization of DSGG from human kidney tissue is ongoing. SGG has been previously isolated,

purified, and definitively characterized as to structure only from a human teratocarcinoma cell line, 2102Ep (11). DSGG has been purified from chicken muscle, human erythrocytes, and kidney tumor tissue, and its structure has been definitively proven to be that shown in Table 1 (1, 18, 20, 31). Previous studies by Karr et al. suggested that histological sections of human kidneys could be stained by a MAb directed against stage-specific embryonic antigen 4 (SSEA-4) and that *E. coli* pJFK102 also bound these kidney sections in the same areas stained by the antibodies (15, 16). SSEA-4 is defined as an epitope staining with a MAb raised against 4- and 8-cell-stage mouse embryos and a human teratocarcinoma cell line (33). Based on MAb MC813-70 staining, SSEA-4 has been identified in undifferentiated human embryonic carcinoma cells and seminomas (30). Agglutination of papain-treated human erythrocytes also occurs with MAb MC813-70, identifying the Luke antigen (38, 39), but the molecule on which the Luke antigen is carried on erythrocytes has not been isolated and structurally characterized. Thus, the antibody staining data previously reported by Karr et al. suggested, but did not prove, that SGG was expressed in human kidney tissue. Our data unequivocally demonstrate the presence of both SGG and DSGG in human kidney tissue.

In conclusion, our studies demonstrate the presence of SGG and DSGG in the human kidney and define SGG as a GSL to which each of the three classes of *pap*-encoded adhesins binds avidly. The biological significance of these findings requires further study, but since *E. coli* isolates bearing P fimbrial adhesins are very strongly associated with renal infection (6), SGG may well play a role in the pathogenesis of acute pyelonephritis. Svanborg and associates have also reported an association between nonsecretor status and an increased likelihood of clinically defined inflammatory responses suggestive of pyelonephritis, such as fever, leukocytosis, and elevated C-reactive protein (23). The presence of SGG in the kidneys of nonsecretors could play a role in predisposing these patients to renal inflammation. Further studies are needed to more extensively define the expression of SGG and DSGG in epithelial tissues throughout the urogenital tract. Our data demonstrate the presence of these compounds in the vagina (36) and kidney; we are currently studying the GSL composition of normal human bladder epithelium, including assaying for the presence of SGG and DSGG. Data derived from these studies will increase our knowledge of bladder glycobiology and may eventually lead to novel preventive strategies for UTI through the use of carbohydrate-based compounds that competitively inhibit bacterial attachment.

REFERENCES

1. **Chien, J.-W., and E. L. Hogan.** 1983. Novel pentahexosyl ganglioside of the globo series purified from chicken muscle. J. Biol. Chem. **258:**10727–10730.
2. **Clegg, C.** 1982. Cloning of genes determining the production of mannose-resistant fimbriae in a uropathogenic strain of *Escherichia coli*. Infect. Immun. **38:**739–744.
3. **Folch, J., M. Lees, and G. H. Sloane Stanley.** 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226:**497–509.
4. **Hakomori, S.-I.** 1981. Glycosphingolipids in cellular interaction, differentiation, and oncogenesis. Annu. Rev. Biochem. **50:**733–764.

5. **Hooton, T. M., P. L. Roberts, and W. E. Stamm.** 1994. Effects of recent sexual activity and use of a diaphragm on the vaginal microflora. Clin. Infect. Dis. **19:**274–278.

6. **Johnson, J. R.** 1991. Virulence factors in *Escherichia coli* urinary tract infection. Clin. Microbiol. Rev. **4:**80–128.

7. **Johnson, J. R., and J. J. Brown.** 1996. A novel multiply primed polymerase chain reaction assay for identification of variant *papG* genes encoding the gal(α1-4)gal-binding PapG adhesins of *Escherichia coli*. J. Infect. Dis. **173:**920–926.

8. **Johnson, J. R., T. A. Russo, J. J. Brown, and A. E. Stapleton.** 1996. *papG* alleles of *Escherichia coli* strains causing first-episode or recurrent acute cystitis in adult women. Clin. Infect. Dis. **23:**920.

9. **Johnson, J. R., A. E. Stapleton, T. A. Russo, F. Scheutz, J. J. Brown, and J. N. Maslow.** 1997. Characteristics and prevalence within serogroup O4 of a J96-like clonal group of uropathogenic *Escherichia coli* O4:H5 containing the class I and class III alleles of *papG*. Infect. Immun. **65:**2153–2159.

10. **Kannagi, R., M. N. Fukuda, and S. Hakomori.** 1982. A new glycolipid antigen isolated from human erythrocyte membranes reacting with antibodies directed to globo-N-tetraosylceramide (globoside). J. Biol. Chem. **257:**4438–4442.

11. **Kannagi, R., S. B. Levery, F. Ishigami, S.-I. Hakomori, L. H. Shevinsky, B. B. Knowles, and D. Solter.** 1983. New globoseries glycosphingolipids in human teratocarcinoma reactive with the monoclonal antibody directed to developmentally regulated antigen, stage-specific embryonic antigen 3. J. Biol. Chem. **258:**8934–8942.

12. **Kannagi, R., E. Nudelman, S. B. Levery, and S. Hakomori.** 1982. A series of human erythrocyte glycosphingolipids reacting to the monoclonal antibody directed to a developmentally regulated antigen, SSEA-1. J. Biol. Chem. **257:**14865–14872.

13. **Kannagi, R., K. Watanabe, and S. Hakomori.** 1987. Isolation and purification of glycosphingolipids by high-performance liquid chromatography. Methods Enzymol. **138:**3–12.

14. **Karlsson, K.-A.** 1989. Animal glycosphingolipids as membrane attachment sites for bacteria. Annu. Rev. Biochem. **58:**309–350.

15. **Karr, J. F., B. Nowicki, L. D. Truong, R. A. Hull, and S. I. Hull.** 1989. Purified P fimbriae from two cloned gene clusters of a simple pyelonephritogenic strain adhere to unique structures in the human kidney. Infect. Immun. **57:**3594–3600.

16. **Karr, J. F., B. J. Nowicki, L. D. Truong, R. A. Hull, J. J. Moulds, and S. I. Hull.** 1990. *pap-2*-encoded fimbriae adhere to the P blood group-related glycosphingolipid stage-specific embryonic antigen 4 in the human kidney. Infect. Immun. **58:**4055–4062.

17. **Kinane, D. F., C. C. Blackwell, R. P. Brettle, D. M. Weir, F. P. Winstanley, and R. A. Elton.** 1982. ABO blood group, secretor state and susceptibility to recurrent urinary tract infection in women. Br. Med. J. **28:**7–9.

18. **Kundu, S. K., B. E. Samuelsson, I. Pascher, and D. M. Marcus.** 1983. New gangliosides from human erythrocytes. J. Biol. Chem. **258:**13857–13866.

19. **Leffler, H., and C. Svanborg-Eden.** 1980. Chemical identification of a glycosphingolipid receptor for *Escherichia coli* attaching to human urinary tract cells and agglutinating human erythrocytes. FEMS Microbiol. Lett. **8:**127–134.

20. **Levery, S. B., M. E. Salyan, S. J. Steele, R. Kannagi, S. Dasgupta, J. L. Chien, E. L. Hogan, H. van Halbeck, and S. Hakomori.** 1994. A revised structure for disialosyl globo-series gangliosides of human erythrocytes and chicken skeletal muscle. Arch. Biochem. Biophys. **312:**125–134.

21. **Lindstedt, R., N. Baker, P. Falk, R. Hull, S. Hull, J. Karr, H. Leffler, C. Svanborg-Eden, and G. Larson.** 1989. Binding specificities of wild-type and cloned *Escherichia coli* strains that recognize globo-A. Infect. Immun. **57:**3389–3394.

22. **Lomberg, H., B. Cedergren, H. Leffler, B. Nilsson, A.-S. Carlstrom, and C. Svanborg-Eden.** 1986. Influence of blood group on the availability of receptors for attachment of uropathogenic *Escherichia coli*. Infect. Immun. **51:**919–926.

23. **Lomberg, H., U. Jodal, H. Leffler, P. De Man, and C. Svanborg.** 1992. Blood group non-secretors have an increased inflammatory response to urinary tract infection. Scand. J. Infect. Dis. **24:**77–83.

24. **Magnani, J. L., D. Smith, and V. Ginsburg.** 1980. Detection of gangliosides that bind cholera toxin: direct binding of ^{125}I-labeled toxin to thin-layer chromatograms. Anal. Biochem. **109:**399–402.

25. **Naoi, M., Y. C. Lee, and S. Roseman.** 1974. Rapid and sensitive determination of sphingosine bases and sphingolipids with fluorescamine. Anal. Biochem. **58:**571–577.

26. **Normark, S., D. Lark, R. Hull, M. Norgren, M. Båga, P. O'Hanley, G. Schoolnik, and S. Falkow.** 1983. Genetics of digalactoside-binding adhesin from a uropathogenic *Escherichia coli* strain. Infect. Immun. **41:**942–949.

27. **Nudelman, E., Y. Fukush, S. B. Levery, T. Higuchi, and S.-I. Hakomori.** 1986. Novel fucolipids of human adenocarcinomas: disialosyl Le^a antigen (III^{4}FucIII^{6}NeuAcIV^{3}NeuAcLc$_4$) of human colonic adenocarcinoma and the monoclonal antibody (FH7) defining this structure. J. Biol. Chem. **261:**5487–5495.

28. **Nudelman, E., R. Kannagi, S. Hakomori, M. Parsons, M. Lipinski, J. Wiels, M. Fellous, and T. Tursz.** 1983. A glycolipid antigen associated with Burkitt lymphoma defined by a monoclonal antibody. Science **220:**509–511.

29. **Nudelman, E. D., U. Mandel, S. B. Levery, T. Kaizu, and S. Hakomori.** 1989. A series of disialogangliosides with binary 2-3 sialosyllactosamine structure, defined by monoclonal antibody NUH2, are oncodevelopmentally regulated antigens. J. Biol. Chem. **264:**18719–18725.

30. **Olie, R. A., B. Fenderson, K. Daley, J. W. Oosterhuis, J. Murphy, and L. H. J. Looijenga.** 1996. Glycolipids of human primary testicular germ cell tumours. Br. J. Cancer **74:**133–140.

31. **Saitoh, S., S. B. Levery, M. E. K. Salyan, R. I. Goldberg, and S. Hakomori.** 1994. Common tetrasaccharide epitope NeuAca2-3Galβ1-3(NeuAca2-6)GalNAc, presented by different carrier glycosylceramides or O-linked peptides, is recognized by different antibodies and ligands having distinct specificities. J. Biol. Chem. **269:**5644–5652.

32. **Sheinfeld, J., A. J. Schaeffer, C. Cordon-Cardo, A. Rogatko, and W. R. Fair.** 1989. Association of the Lewis blood-group phenotype with recurrent urinary tract infections in women. N. Engl. J. Med. **320:**773–777.

33. **Shevinsky, L. H., B. B. Knowles, I. Damjanov, and D. Dolter.** 1982. Monoclonal antibody to murine embryos defines a stage-specific embryonic antigen expressed on mouse embryos and human teratocarcinoma cells. Cell **30:**697–705.

34. **Siddiqui, B., and S. Hakomori.** 1970. Change of glycolipid pattern in Morris hepatomas 5123 and 7800. Cancer Res. **30:**2930–2936.

35. **Stapleton, A., S. Moseley, and W. E. Stamm.** 1991. Urovirulence determinants in *Escherichia coli* isolates causing first-episode and recurrent cystitis in women. J. Infect. Dis. **163:**773–779.

36a.**Stapleton, A. E., M. R. Stroud, S. I. Hakomori, and W. E. Stamm.** 1995. Uropathogenic *Escherichia coli* bind with highest affinity to the globo-series glycosphingolipid sialosyl galactosyl globoside. Clin. Infect. Dis. **21:**727. (Abstract.)

36. **Stapleton, A., E. Nudelman, H. Clausen, S.-I. Hakomori, and W. E. Stamm.** 1992. Binding of uropathogenic *Escherichia coli* R45 to glycolipids extracted from vaginal epithelial cells is dependent on the histo-blood group secretor status. J. Clin. Invest. **90:**965–972.

37. **Stromberg, N., B.-I. Marklund, B. Lund, D. Ilver, A. Hamers, W. Gaastra, K.-A. Karlsson, and S. Normark.** 1990. Host-specificity of uropathogenic *Escherichia coli* depends on differences in binding specificity to galα1-4 gal-containing isoreceptors. EMBO J. **9:**2001–2010.

37a.**Stroud, M. R., A. E. Stapleton, and S. B. Levery.** Submitted for publication.

38. **Tippett, P., P. W. Andrews, B. B. Knowles, D. Solter, and P. N. Goodfellow.** 1986. Red cell antigens P (globoside) and Luke: identification by monoclonal antibodies defining the murine stage-specific embryonic antigens -3 and -4 (SSEA-3 and SSEA-4). Vox Sang. **51:**53–56.

39. **Tippett, P., R. Sanger, R. R. Race, J. Swanson, and S. Busch.** 1965. An agglutinin associated with the P and the ABO blood group systems. Vox Sang. **10:**269–280.

40. **Watanabe, K., and Y. Arao.** 1981. A new solvent system for the separation of neutral glycosphingolipids. J. Lipid Res. **22:**1020–1024.

41. **Yu, R. K., and R. W. Ledeen.** 1972. Gangliosides of human, bovine, and rabbit plasma. J. Lipid Res. **13:**680–686.

The P Histo-Blood Group-Related Glycosphingolipid Sialosyl Galactosyl Globoside as a Preferred Binding Receptor for Uropathogenic Escherichia coli: Isolation and Structural Characterization from Human Kidney†

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[1]Abbreviations: Cer, ceramide; C/M, chloroform/methanol; Fuc, L-fucose; Gal, D-galactose; Glc, D-glucose; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine; ^{1}H-NMR, proton nuclear magnetic resonance; HPTLC, high performance thin-layer chromatography; HPLC, high performance liquid chromatography; IHW, isopropyl alcohol-hexane-water; MAb, Monoclonal antibody; NeuAc, *N*-acetylneuraminic acid; PBS, phosphate buffered saline; SGG, sialosyl galactosyl globoside; SSEA-4, stage specific embryonic antigen 4; UTI, urinary tract infection. Glycolipids are abbreviated according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [*Lipids* (1977) *12*, 455-463]; however the suffix -OseCer is omitted.

[2]The nomenclature of Domon and Costello, 1988, will be used here, with the following modifications: 1) the sodiated "B" ion, which formally corresponds to B−H+Na, will be referred to as β•Na; this can be justified by analogy to older nomenclature for the reaction thought to produce the neutral analog of this fragment in FAB/SIMS processes— the β-elimination; see Dell, 1987; 2) the numbering of the cleavage sites will correspond to the residue designations used in discussing the NMR spectrum, proceeding only in one direction from the non-reducing end, and carrying the Roman numeral and capital letter subscripts to avoid confusion with the numbering system of Domon and Costello, 1988).

[3]Levery, S. B., manuscript in preparation.

The P histo-blood group-related glycosphingolipid, sialosyl galactosyl globoside (SGG), has recently been implicated as a preferred binding receptor for uropathogenic *Escherichia coli* [Stapleton, A.E., Stroud, M.R., Hakomori, S., and Stamm, W.E. (1998) *Infect. Immun.* In Press]. We report here the purification and complete structural characterization of SGG from normal human kidney. Using metabolically [^{35}S]-labeled *E. coli* as a probe, a monosialylated glycosphingolipid was isolated to homogeneity. The glycosphingolipid was purified by a combination of high performance liquid chromatography and preparative high performance thin-layer chromatography and its structure unambiguously elucidated by ^{1}H-NMR, electrospray ionization mass spectrometry, and methylation analysis. Its primary structure was shown to be identical to a previously characterized, developmentally regulated, globo-series glycolipid thought to be unique to human teratocarcinoma. The significance of this structure as a unique receptor in human kidney for uropathogenic *E. coli* and its role in the pathogenesis of urinary tract infections is discussed.

INTRODUCTION

Nearly all of the antigenic determinants defining the P histo-blood group system (P^k, P, LKE, and P_1) are associated with globo-series glycosphingolipids (Race and Sanger, 1975; Marcus, et. al., 1981). Globo-series glycosphingolipids (GSLs) are defined at the chemical level as having the trisaccharide Galα1→4Galβ1→4Glcβ1 (P^k) attached at the reducing end to ceramide (*N*-fatty acyl sphingosine). This "globo-core" can be further glycosylated to form more complex carbohydrate structures that define additional P blood group related antigens, i.e., P and LKE (Tippett et. al., 1986), as well as those antigens associated with the ABO histo-blood group system (Clausen and Hakomori, 1989). P blood group antigenic structures are strictly carried on glycosphingolipids (Yang et al., 1994) and play an important role in the pathogenesis of urinary tract infections, particularly as receptors for uropathogenic *Escherichia coli* (Korhonen et. al., 1982). The Galα1→4Gal sequence found in all blood group P antigens is the minimal structure required for binding P fimbria-expressing uropathogenic *E. coli* (Källenius et. al., 1980; Leffler and Svanborg-Edèn, 1980). However, women expressing P blood group related antigens who carry ABH blood group antigens in secretions (secretors) have a lower incidence of urinary tract infections (UTI) than individuals (nonsecretors) who do not express these determinants (Kinane, et al.,1982; Sheinfeld, et al., 1989; Lomberg, et al., 1986). It has been hypothesized that the increased risk of UTI in nonsecretors and the increased attachment of uropathogenic bacteria to their uroepithelial cells (Lomberg, et al., 1986) is due to the expression of a unique receptor for *E. coli* (Stapleton, et al., 1992). Evidence suggests that nonsecretors selectively express two extended globo-series GSLs, sialosyl galactosyl globoside (SGG) and disialosyl galactosyl globoside (DSGG) which specifically bind uropathogenic *E. coli* (Stapleton et al, 1992) and that one of these GSLs (SGG) is the preferred receptor over other globo-series GSLs for *E. coli* isolates expressing a P-related adhesin (Stapleton, et al., 1998).

Since globo-series glycolipids have been shown to be modified by histo-blood group status, and the globo-core is the major carrier isotype (type 4 chain) of ABH active epitopes in renal epithelium (Breimer and Jovall, 1985), we hypothesized that the increased risk of UTI in nonsecretors may be due to the presence of a unique receptor for

P-fimbriated *E. coli* in these patients. In the present study, using a metabolically [^{35}S]-labeled clinical *E. coli* isolate (R45) as a probe, a monosialylated ganglioside comigrating with a sialosyl galactosyl globoside standard by high performance thin-layer chromatography (HPTLC) in two different solvent systems was purified from normal human kidney. Its structure was unambiguously confirmed by ^{1}H-NMR, electrospray ionization mass spectrometry (ESI-MS), and methylation analysis as V^{3}NeuAcGb$_5$Cer (SGG), a developmentally regulated globo-series glycosphingolipid previously thought to be unique to human teratocarcinoma and sharing the same terminal epitope as the stage-specific embryonic antigen, SSEA-4. To our knowledge, this is the first report describing the complete and unambiguous structural characterization of SGG from normal human tissue. Its identification in human kidney, a target organ for uropathogenic *E. coli* infection, and its role as a high affinity ligand (Stapleton, et al., 1998) may explain the chemical basis for the increased risk of UTI in nonsecretors.

MATERIALS AND METHODS

Preparation of human kidney ganglioside fraction. (A) Glycolipid extraction. Approximately 800 grams of pooled human kidney tissue was extracted by homogenization (Kannagi, et al., 1982) in a Waring blender with 10 volumes of the lower phase of Isopropanol:Hexane:Water (IHW; 55:25:20). The extract was filtered through a Whatmann No. 1 filter and the residue re-extracted and filtered as above. The extraction/filtration procedure was repeated once more, and the combined filtrates were concentrated under reduced pressure at 40°C using a Brinkman rotary evaporator. The concentrated extract was subjected to Folch partitioning by dissolving the residue in 3 L of C/M (2:1) containing 500 mL water. After vigorous shaking, the extract was allowed to separate until two translucent phases appeared (Folch, et al., 1957). The upper phase was removed and the lower phase re-extracted by the addition of C/M/1% KCl (1:10:10) to the original level. The liquid-liquid extraction procedure was repeated two times, and the combined upper phases were concentrated by rotary evaporation, reconstituted in water, and dialyzed exhaustively against deionized water using Spectropor 3 dialysis tubing (MW cutoff = 3500).

(B) Anion Exchange Chromatography. After dialysis, the upper-phase extract was evaporated to dryness as above and dissolved in 50 mL of C/M/water (30:60:8) by a combination of warming at 37°C and sonication. Insoluble material was removed by centrifugation at 1000g for 10 min and re-extracted by sonication in an additional 50 mL of the same solvent. Following centrifugation as above, the combined supernatants were loaded onto a DEAE-sephadex column (300 mL bed volume; acetate form) and washed with 2 L of C/M/water (30:60:8) to remove all neutral lipids (Yu and Ledeen, 1972). The column was equilibrated with 500 mL of methanol and the ganglioside fraction eluted with 2 L of 1.0 M NH_4OAc in methanol. The eluted ganglioside fraction was dried by rotary evaporation, dialyzed against water, and dried as above.

Purification of sialosyl galactosyl globoside from human kidney ganglioside fraction. (A) High performance liquid chromatography. The ganglioside fraction was solubilized in 10 mL of IHW and transferred from the evaporation flask to a 15 mL tube. The sample was completely dried under N_2 at 37°C using a nitrogen evaporator (N-EVAP, Organomation Inc., South Berlin, MA) and reconstituted in 2 mL of IHW by sonication. The sample was injected onto a preparative Iatrobead column (6RS-8010; 0.8 x 60 cm; Iatron Laboratories Inc., Kanda/Tokyo, Japan) pre-equilibrated with IHW (55:40:5) and subjected to a linear gradient from IHW 55:40:5 to 55:25:20 with a flow rate of 1 mL/min (Ando, et al., 1976). Four mL fractions were collected over 400 min. Each fraction was spotted onto an HPTLC plate, developed in chloroform/methanol/0.5%CaCl₂ (described below), and visualized by spraying with 0.5% orcinol in 2 N sulfuric acid. A parallel plate was developed and used in bacterial overlay assays (see below). Fractions staining positive to orcinol and *E. coli* were pooled according to migration. HPLC fractions 39-48, showing the strongest binding by the *E. coli* overlay assay and containing more than one band by TLC were pooled, dried under N_2, resolubilized in 1mL of IHW, and injected onto a semipreparative Iatrobead column (0.4 x 60 cm). A linear gradient from IHW 55:40:5 to 55:25:20 over 200 min with a flow rate of 0.5 mL/min was used. One milliliter fractions were collected, assayed, and pooled as described above. One pool (fractions 53-58), showing the strongest staining by

E. coli and containing multiple bands by TLC was further purified by preparative HPTLC (described below).

(B) *High Performance TLC.* The strongest staining band in fraction 53-58 was separated by preparative HPTLC in chloroform/methanol/0.5%CaCl$_2$ (50:40:10) so as only a single orcinol and *E. coli* positive band was observed. The sample was further resolved into three additional bands by HPTLC using a solvent system of 1-propanol/water/NH$_4$OH (6:1.5:1). Preparative TLC was performed by streaking 50μL of sample across a 10 x 20 cm HPTLC silica gel plate (silica gel 60; EM Science, Gibbstown, NJ), drying, and developing in the appropriate solvent system. Plates were dried, and bands were visualized by spraying with 0.03% primulin in 80% acetone. Bands were marked with a pencil under UV light. Marked bands were scraped from the plate using a razor blade, and the gangliosides were extracted from the silica by sonicating for 20 min in IHW (55:25:20; 2 mL per band). The silica was removed by centrifuging at 1000g for 10 min and re-extracted as above, and the combined supernatants were dried under N$_2$. Samples were cleaned up using 1 cm^3 tC-18 Sep-Pak cartridges (Waters, Milford, MA) by first dissolving the sample in 1mL of PBS and then applying it to a column equilibrated with PBS after sequentially washing with 5 mL of methanol and 5 mL of water. Once the sample was retained, the column was washed with 10 mL of water, followed by 10 mL of 50% methanol, and eluted in 5 mL of 100% methanol. The sample was dried under N$_2$, dissolved in 1 mL IHW (55:25:20), and injected onto an Iatrobead column (0.4 x 30 cm) as above using a linear gradient from IHW 55:40:5 to 55:25:20 for 100 min at a flow rate of 1 mL/min. One milliliter fractions were collected and visualized by HPTLC using the orcinol-sulfuric acid reaction. Orcinol-positive fractions were pooled and dried under N$_2$ prior to structural analysis.

Bacterial overlay assays. Assays were performed as previously described (Stapleton, et al., 1992) using metabolically [^{35}S]-labeled *E. coli* isolate R45, a wild-type cystitis isolate (Stapleton, et al., 1991) which expresses P fimbriae carrying the Class II *pap*-encoded adhesin (Johnson, et al., 1997) and binds globo-series glycolipids.

¹H-nuclear magnetic resonance spectroscopy. A sample of the ganglioside was prepared for NMR analysis by repeated lyophilization from D_2O (99.996 atom %; Cambridge Isotope Laboratories, Woburn, MA), then dissolved in 0.5 mL DMSO-d_6 (99.96 atom %; Aldrich, Milwaukee, WI) containing 2 % D_2O. All ¹H-NMR spectra were acquired at 600 MHz on a Bruker (Karlsruhe, Germany) DRX-600 Fourier transform spectrometer, at a probe temperature of 308°K, a sweep width of 3600 Hz, and with suppression of the residual HOD resonance by a presaturation pulse during the preparatory delay period. 1-D ¹H-NMR spectra were resolution enhanced by exponential multiplication (LB=0.3) prior to Fourier transformation. Acquisition and processing of 2-D TPPI-COSY, -TOCSY, and -NOESY experiments were performed essentially as described previously (Levery *et al.*, 1992, and references cited therein).

Positive ion mode electrospray ionization mass spectrometry. ESI-MS experiments were carried out on a PE-Sciex API-III spectrometer with IonSpray source. A sample of ganglioside was permethylated as described previously (Levery & Hakomori, 1987), dissolved in methanol containing 1 mM ammonium acetate, and introduced by direct infusion. For single quadrupole spectra, the mass range *m/z* 100-2200 was scanned at an orifice-to-skimmer potential of 180 V; or, for a higher resolution spectrum of the pseudomolecular ion region, *m/z* 2000-2250 at 200 V. For tandem ESI-MS/CID-MS experiments, the orifice-to-skimmer potential was lowered to 120 V to increase the abundance of disodiated, doubly charged pseudomolecular ions; argon was introduced into the collision cell at CGT≅400, and precursor ions were selected in Q_1, while the mass range *m/z* 100-2200 was scanned in Q_3.

Linkage analysis by GC-MS. An aliquot of permethylated ganglioside was depolymerized, reduced, and acetylated essentially as described (Levery & Hakomori, 1987). Analysis of the resultant partially methylated alditol acetates (PMAAs) was performed on a Hewlett-Packard 5890 GC/5970 MSD operating in the splitless mode, using a 30 m DB-5 bonded phase fused silica capillary column, temperature programmed from 160-260°C at 2°C/min. PMAA derivatives were identified by retention times and characteristic EI mass spectra (Bjorndal *et al.*, 1970; Jansson *et al.*, 1976) compared with those of authentic standards.

RESULTS

Purification of SGG from normal human kidney. The HPLC elution pattern of *E. coli*-binding gangliosides as determined by HPTLC is shown in Figure 1. Fractions 39 to 48 were pooled based on strong binding to [^{35}S]-labeled *E. coli* and comigration with an SGG standard prepared from partially desialylated DSGG. Pooled fraction 39-48 was subjected to a second HPLC as described in Materials and Methods. Fractions 53-58 obtained after the second HPLC were pooled based on the same criteria described above. A single band comigrating with the SGG standard in C/M/0.5%CaCl$_2$ (50:40:10) was obtained after preparative HPTLC in the same solvent system (data not shown). The GSL sample was further resolved into three components and purified by preparative TLC in a solvent system consisting of 1-propanol/water/NH$_4$OH (6:1.5:1). A thin-layer chromatogram showing the separation of the three GSL components after preparative TLC in the above solvent system is shown in Figure 2A. The three GSL fractions were labeled according to their migration in 1-propanol/water/NH$_4$OH (6:1.5:1); i.e., the fastest migrating component was labeled #1 and the slowest #3. All three samples were rechromatographed in C/M/0.5%CaCl$_2$ (50:40:10) on duplicate plates. Figure 2B shows a thin-layer chromatogram stained for carbohydrates by the orcinol-sulfuric acid reaction. Figure 2C shows the same chromatogram as in Figure 2B with the bands revealed by [^{35}S]-labeled *E. coli*. Fraction #3 which comigrated with the SGG standard (Figure 2B) and showed the strongest binding by *E. coli* (Figure 2C) was subjected to structural analysis.

^{1}H-NMR spectroscopy. Portions of the 1-D ^{1}H-NMR spectrum of the monosialosyl ganglioside from human kidney are reproduced in Figure 3. Although a number of signals from glycosphingolipid and other impurities can be observed, the spectrum of the major component is clearly similar to that obtained previously from V^{3}NeuAcGb$_5$Cer (GL-7) originally isolated from the human teratocarcinoma cell line 2102Ep (Kannagi et al, 1983). With respect to anomeric and other structural reporter resonances, the chemical shift differences are all < 0.01 ppm. The sugar residues of the core glycan are represented by five anomeric resonances, four β- ($^3J_{1,2}$ = 7-9 Hz) and one

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α- ($^3J_{1,2}$ = 2-4 Hz), as expected for a Gb$_5$ pentasaccharide. The chemical shifts of two β-anomeric signals (4.185 and 4.256 ppm) and the single α-anomeric signal (4.815 ppm) are very close to those of β-Glc I, β-Gal II, and α-Gal III, respectively, in Gb$_4$Cer and Gb$_5$Cer measured under similar conditions (Dabrowski et al, 1980a; Kannagi et al, 1983). The remaining β-anomeric resonances (4.579 and 4.234 ppm) correspond to those for β-GalNAc IV and β-Gal V, respectively, in Gb$_5$Cer, with a relative upfield shift for the former (-0.03 ppm) and a downfield shift for the latter (+0.04 ppm). These are the glycosylation-induced shift changes expected for addition of NeuAcα2→3 to a terminal Galβ1→3/4HexNAc group (Koerner et al, 1983; Levery et al, 1988). The additional NeuAc residue is distinguished by an H-3eq signal (2.766 ppm, dd) and an NAc signal (1.889 ppm, s, 3H) at chemical shifts diagnostic for an α2→3 linkage to terminal β-Gal (Koerner et al, 1983; Levery et al, 1988). A second NAc signal (1.795 ppm, s, 3H) could be assigned to the β-GalNAc residue; it was observed previously at 1.797 ppm in the spectrum of V^{3}NeuAcGb$_5$Cer (Levery et al, 1994).

In order to substantiate the proposed glycan structure, as well as confirm the proton resonance assignments, the sample was subjected to a series of 2-D NMR experiments, including PS-DQF-COSY, TOCSY (see Figure 4), and NOESY. This allowed assignment of H-1 through H-4 of all three β-*galacto*pyranoside spin systems, H-1 through H-6 of both the α-*galacto*pyranoside and the β-*gluco*pyranoside spin systems, and H-3 through H-5 of the NeuAc spin system, along with the functionalized proximal part of the ceramide spin system. Because of the vanishingly-small $^3J_{4,5}$ coupling in galactopyranosides, which attenuates transmission of coherence between H-4 and H-5 of a *galacto*pyranoside (Inagaki et al, 1987, 1989; Dabrowski et al, 1988), it was not practical to assign H-5 and H-6 resonances for these spin systems, except in the case of α-Gal III, where the distinct downfield position of H-5 allows a connectivity to be established with the remaining ring protons in the TOCSY experiment. The assignments, the majority of which have not been available previously for this compound, are summarized in Table 2. The glycan primary structure was further substantiated by detection of the following dipolar interactions between glycosidically-linked residues in the NOESY experiment: V-1/IV-3; IV-1/III-3; III-1/II-4; and II-1/I-4. Although no

interglycosidic dipolar interactions were detected originating from the NeuAc residue, the NeuAcα2→3 linkage to terminal β-Gal was confirmed by the diagnostic positions observed for V-3, V-4, and A-3ax (Koerner et al, 1983; Levery et al, 1988).

The complex of resonances corresponding to the major ceramide component indicated that it is composed of sphing-4-enine and non-α-hydroxylated fatty acids (Dabrowski et al, 1980b; Koerner et al, 1983). Only a small amount of fatty acid unsaturation is indicated by the low amplitudes of the cis-vinyl proton signal at 5.320 ppm and the allyl proton signal at 1.976 ppm. The presence of a ceramide component containing α-hydroxylated fatty acids is indicated by the observation of additional β-Glc H-1 and H-2 resonances at 4.215 and 3.026 ppm, respectively (Levery et al, 1988).

Permethylation and ESI-MS. Following permethylation, a portion of the material was subjected to analysis by electrospray ionization mass spectrometry in the positive ion mode. Single quadrupole analysis at high orifice-to-skimmer potentials (150-200 V) gave abundant singly charged [M•Na]$^+$ and doubly charged [M•2Na]$^{2+}$ pseudomolecular ion species. The overall abundances, as well as the ratio, of these species depended on the potential used, although they were not always strictly reproducible even at a given potential due to the influence of other variables. High potentials resulted in significant and useful glycosidic fragmentation, yielding ions of the B- and [C•Na]-type, along with some of the [β•Na]- and [Y/C•Na]-type ions[2] normally seen under ESI-MS/CID-MS conditions. An additional set of ions corresponding to [C•Na+CH$_2$] were observed for a number of glycosidic cleavages; the precise origin of these fragments will be discussed elsewhere[3].

The monosodiated pseudomolecular ion series (see Figure 5; Scheme 1) corresponded to a glycan formula NeuAc•Hex$_4$•HexNAc attached to ceramides consisting of d18:1 sphingosine in combination with fatty acids 16:0-24:0, with 22:0 and 24:0 predominating. The presence of significant amounts of h22:0 and h24:0 fatty acids was also apparent from pseudomolecular ions 30 u higher in mass (in the former case, the ion at *m/z* 2138 is poorly resolved from that at *m/z* 2136). The fragmentation clearly supports the linear glycan sequence proposed for the ganglioside, as shown in Scheme 1. Although the lack of an observable B$_V$ ion at m/z 580 means that the NeuAc residue can't

be definitively placed on the terminal Hex on the basis of this series alone, the combination of internal glycan ions $[Y_V/C_I \bullet Na]$ (m/z 1102) and $[Y_{IV}/C_I \bullet Na]$ (m/z 898) is supportive. However, the latter ion is of rather low abundance. ESI-MS/CID-MS experiments, as described below, were more definitive.

Triple quadrupole ESI-MS/CID-MS experiments were carried out under conditions maximizing the yield of disodiated doubly charged pseudomolecular ions. The most abundant of these were selected by Q_1 and collisionally activated in Q_2 while scanning Q_3. The results of three of these experiments are reproduced in Figure 6. The spectra are characterized by an abundance of glycosidic cleavage fragments of the $[\beta \bullet Na]$-, $[C \bullet Na]$-, $[Y \bullet Na]$- $[Y/\beta \bullet Na]$- and $[Y/C \bullet Na]$-types (see Scheme 2). Of particular significance is the abundant ion m/z 620, corresponding to the $[C_V \bullet Na]$ fragment which clearly shows the attachment of NeuAc to the terminal Hex residue.

Linkage analysis by GC-MS. The remainder of the permethylated ganglioside was depolymerized, reduced, and acetylated according to standard procedures, and the resulting partially methylated alditol acetates (PMAAs) analyzed by GC-EI-MS. PMAAs identified by their retention times and characteristic EI spectra were 2,3,6-tri-O-Me-Gal ($\rightarrow$4Gal); 2,4,6-tri-O-Me-Gal ($\rightarrow$3Gal); 2,3,6-tri-O-Me-Glc ($\rightarrow$4Glc); and 4,6-di-O-Me-GalNAcMe ($\rightarrow$3GalNAc). Detection of these derivatives confirmed unambiguously all of the linkages proposed on the basis of the NMR analysis, and, together with the residue sequence derived from ESI-MS data, confirms that the complete primary structure is V^3NeuAcGb$_5$Cer.

DISCUSSION

Globo-series glycosphingolipids are characterized by an internal Galα1$\rightarrow$4Gal sequence and are found in the outer leaflet of the plasma membrane where they are exposed to the extracellular environment (Hakomori, 1981). The expression of globo-series glycolipids is determined by the P histo-blood group system (Race and Sanger, 1975; Marcus et. al., 1981) and plays an important role in the pathogenesis of UTI by mediating the attachment of *E. coli* to uroepithelial cells. Uropathogenic *E. coli* bind to P blood group antigens via *pap* gene encoded fimbrial adhesins, hence the name P fimbriae

(Källenius, et. al., 1981; Korhonen, et. al., 1982). In addition to the role of P blood group antigens in the pathogenesis of UTI, they have also been shown to act as receptors for verotoxins (Jacewicz et. al., 1986; Lindberg, et. al. 1987) and parvoviruses (Brown, et. al., 1993), and antibodies directed against some of these antigens (P and P^k) have been implicated in spontaneous abortion (Lopez et. al., 1983; Shirey et. al., 1987; Hansson et. al., 1988) as well as the rare autoimmune disorder paroxysmal cold hemoglobinuria (Schwarting, et. al., 1979).

SGG is a sialylated extended form of globo-series glycolipid first reported as a developmentally regulated antigen isolated and structurally characterized from the human teratocarcinoma cell line 2102Ep (Kannagi et al., 1983) and subsequently identified in chicken pectoral muscle (Chien and Hogan, 1983). This antigen contains a terminal trisaccharide epitope (NeuAcα2→3Galβ1→3GalNAc) defined by a monoclonal antibody directed to murine stage specific embryonic antigen 4 (SSEA4). In addition to forming the terminal epitope of SGG, this trisaccharide is also found on extended ganglio-series glycolipids, i.e., GM$_{1b}$, GD$_{1a}$, and GT$_{1b}$, as well as on sialosyl galactosyl-A (Clausen, et. al., 1989) and the monosialylated derivative of the T-antigen (Thomsen-Friedenreich antigen) present on O-linked glycoproteins.

In our previous study, glycosphingolipids were extracted from vaginal epithelial cells collected from women who were nonsecretors and secretors of ABO blood group antigens (Stapleton et. al., 1992). A radiolabeled *E. coli* isolate (R45) bound to two glycolipids present in the extract derived from nonsecretors but not from secretors when separated on TLC plates. These glycolipids comigrated with SGG and DSGG standards and the higher migrating glycolipid (comigrating with SGG) stained positive with a monoclonal antibody that binds SGG. The presence of SGG in tissues of nonsecretors is consistent with the idea that an α2→3sialyltransferase preferentially sialylates the precursor galactosyl globoside in the absence of the α1→2fucosyltransferase encoded by the *Se* gene.

The data presented in this report describe for the first time the purification and unambiguous structural characterization of SGG from human kidney and support the hypothesis that SGG is an important physiological receptor for uropathogenic *E. coli*

expressing *pap*-encoded adhesins. In a parallel study, we demonstrated that this human kidney derived SGG binds to wild type uropathogenic *E. coli* strain R45 in addition to cloned *pap* adhesin-expressing *E. coli* strains representing all three classes of known P fimbrial adhesins (Stapleton et al, 1998). More important, all strains of *E. coli* assayed bind to SGG with high avidity when compared to closely related globo-series glycosphingolipids.

Earlier studies by Karr et. al., showed that *pap-2*-encoded P-fimbria purified from a pyelonephritic strain of *E. coli* bound to cryostat sections of human kidney and to human erythrocytes expressing the Luke (LKE) antigen (defined by anti-LKE serum) and that binding to kidney sections could be inhibited by preincubation with a monoclonal antibody to SSEA4 (defined by MAb MC813-70). They concluded that the receptor for the *pap-2* encoded P-fimbria was LKE on human erythrocytes and SSEA4 on human kidney. Although it has been suggested that LKE and SSEA4 are identical antigens, the relationship is limited to a common determinant recognized by both anti-LKE serum and MAb MC813-70. Regardless of the fact that this determinant is found on SGG, the presence of this glycolipid in human kidney or any other normal human tissue has until now only been speculative. A recent study described the fine specificity of a monoclonal antibody directed to DSGG (Saito et. al., 1994). This MAb is able to discriminate between a common branched tetrasaccharide epitope (NeuAcα2→3Galβ1→3{NeuAcα2→6}GalNAc) found on DSGG, GD1α, and a common mucin-type epitope widely distributed on glycoproteins such as glycophorin A. The differential antibody-binding ability of this structure is thought to be dependent on its carrier glycoconjugate and branched characteristics. Monoclonal antibodies specific for globo-series ABH antigens (Bremer, et. al., 1984; Clausen, et. al., 1986) as well as SGG are unavailable, and the difficulty in generating specific MAbs to these structures may be due to their linear characteristics. Unfortunately, until such MAbs are available, studies involving their tissue distribution will be extremely difficult. The results of this investigation clearly illustrate the presence of SGG in human kidney and may suggest a chemical basis for the increased risk of UTI in nonsecretors.

ACKNOWLEDGEMENTS

REFERENCES

1. Race R.R. and Sanger R. (1975) *Blood Groups in Man*, 6th ed. Blackwell Scientific Publications, Oxford

2. Marcus, D.M., Kundu, S.K., and Suzuki, A. (1981) *Semin. Hematol. 18*, 63-71

3. Tippett, P., Andrews, P.A., Knowles, B.B., Solter, D., and Goodfellow, P.N. (1986) *Vox Sang. 51*, 53-56

4. Clausen, H. and Hakomori, S. (1989) *Vox Sang. 56*, 1-20

5. Yang, Z., Bergström, J., and Karlsson, K-A. (1994) *J. Biol. Chem. 269*, 14620-14624.

6. Korhonen, T.K., Väisänen, V., Saxén, H., Hultberg, H., and Svenson, S.B. (1982) *Infect. Immun. 37*, 286-291

7. Källenius, G., Möllby, R., Svenson, S.B., Winberg, J., and Hultberg, H. (1980) *Infection 8 (Suppl 3)*, 288-293

8. Leffler, H., and Svanborg-Edèn C. (1980) *FEMS Microbiol. Lett. 8*, 127-134

9. Kinane, D.F., Blackwell, C.C., Brettle, D.M., and Wier, F.P. (1982) *Br. Med. J. 285*, 7-9

10. Sheinfeld, J., Schaeffer, A.J., Cordon-Cardo, C., Rogatko, A. and Fair, W.R. (1989) *N. Engl. J. Med. 320*, 773-777

11. Lomberg, H., Cedergren, B., Leffler, H., Nilsson, B., Carlstrom, A.-S. and Svanborg-Eden, C. (1986) *Infect. Immun. 51*, 919-926

12. Stapleton, A., Nudelman, E., Clausen, H., Hakomori, S., and Stamm, W.E. (1992) *J. Clin. Invest. 90*, 965-972

13. Stapleton, A.E., Stroud, M.R., Hakomori, S., and Stamm, W.E. (1998) *Infect. Immun.* In Press.

14. Kannagi, R., Nudelman, E., Levery, S.B., Hakomori, S. (1982) *J. Biol. Chem. 257*, 14865-14874

15. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) *J. Biol. Chem. 226*, 497-509

16. Yu, R.K. and Ledeen, R.W. (1972) *J. Lipid Res. 13*, 680-686

17. Ando, S., Isobe, M., and Nagai, Y. (1976) *Biochim. Biophys. Acta 424*, 98-105

18. Stapleton, A., Mosely, S. and Stamm, W.E. (1991) *J. Infect. Dis. 163*, 773-779

19. Johnson, J.R., Stapleton, A.E., Russo, T.A., Scheutz, F., Brown, J.J., and Maslow, J.N. (1997) *Infect. Immun. 65*, 2153-2159

20. Levery, S.B., Holmes, E.H., Harris, D.D., and Hakomori, S. (1992) *Biochemistry 31*, 1069-1080

21. Levery, S.B., and Hakomori, S., (1987) *Methods. Enzymol. 138E*,13-25

22. Bjorndal, H., Hellerqvist, C.G., Lindberg, B., Svensson S (1970) *Angew. Chem. Intl. Ed. Engl. 9*, 610-619

23. Jansson, P-E., Kenne, L., Liedgren, H., Lindberg, B., and Lonngren, J. (1976) *Chem. Commun.* Univ Stockholm *8*, 1-75

24. Kannagi, R., Levery, S.B., Ishigami, F., Hakomori, S., Shevinsky, L.H., Knowles, B,B., and Solter, D. (1983) *J. Biol. Chem. 258*, 8934-8942

25. Dabrowski, J., Hanfland, P., and Egge, H. (1980a) *Biochemistry 19*, 5652-5658

26. Koerner, T.A.W., Prestegard, J.H., Demou, P.C., and Yu, R.K. (1983) *Biochemistry 22*, 2676-2687

27. Levery, S.B., Nudelman E., Kannagi, R., Symington, F.W., Andersen, N.H., Clausen, H., Baldwin, M., and Hakomori, S. (1988) *Carbohydr. Res. 178*, 121-144

28. Levery, S.B., Salyan, M.E.K., Steele, S.J., Kannagi, R., Dasgupta, S., Chien, J-L., Hogan, E.L., van Halbeek, H., and Hakomori, S. (1994) *Arch. Biochem. Biophys. 312*:125-134.

29. Inagaki, F., Kohda, D., Kodama, C., and Suzuki, A. (1987) *FEBS Lett. 212*, 91-97

30. Inagaki, F., Shimada, I., Kohda, D., Suzuki, A., and Bax, A. (1989) *J. Magn. Reson. 81*, 186-190.

31. Dabrowski, J., Dabrowski, U., Kordowicz, M., and Hanfland, P. (1988) *Biochemistry 27*, 5149-5155.

32. Dabrowski, J., Egge, H., and Hanfland, P. (1980b) *Chem Phys Lipids 26*, 187-196

33. Domon, B., and Costello, C.E. (1988) *Glycoconj. J. 5*, 397-409

34. Dell, A. (1987) *Adv. Carbohydr. Chem. Biochem. 45*, 19-72

35. Hakomori, S. (1981) *Annu. Rev. Biochem. 50*, 733-64

36. Källenius, G., Svenson, S.B., Möllby, R., Cedergren, B., Hultberg, H., and Windberg, J. (1981) *Lancet ii*, 604-606

37. Jacewicz, M., Clausen, H., Nudelman, E., Donohue-Rolf, A., and Keusch, G.T. (1986) *J. Exp. Med. 163*, 1391-1404

38. Lindberg, A.A., Brown, J.E., Stromberg, N., Westling-Ryd M., Schultz, J.E., and Karlsson, K.A. (1987) *J. Biol. Chem. 262*, 1779-1785

39. Brown, K.E., Anderson, S.M., and Young, N.S. (1993) *Science 262*, 114-117

40. Lopez, M., Cartron, J., Cartron, J.P., Mariotti, M., Bony, V., Salmon, C., and Levene, C. (1983) *Clin. Immunol. Immunopathol. 28*, 296-303

41. Shirey, R.S., Ness, P.M., Kickler, T.S., Rock, J.A., Callan, N.A., Schlaff, W.D., and Niebyl, J. (1987) *Transfusion 27*, 189-191

42. Hansson, G., Wazniowska, K., Rock, J.A., Ness, P.M., Kickler, T.S., Niebyl, J.R., and Zopf, D. (1988) *Arch. Biochem. Biophys. 260*, 168-176

43. Schwarting, G.A., Kundu, S.K., and Marcus, D.M. (1979) *Blood 53*, 186-192

44. Chien, J.-L. & Hogan, E.L. (1983) *J. Biol. Chem. 258*, 10727-10730

45. Clausen, H., Levery, S.B., Salyan, M.E.K., Nudelman, E.D., Stroud, M.R., and Hakomori, S. (1989) *J. Biol. Chem. 262*, 14228-14234

46. Breimer, M.E., and Jovall, P.-A. (1985) *FEBS Lett. 176*, 165-172

47. Karr, J.F., Nowicki, B.J., Truong, L.D., Hull, R.A., Moulds, J.J., and Hull, S.I. (1990) *Infect. Immun. 58*, 4055-4062

48. Saito, S., Levery, S.B., Salyan, M.E.K., Goldberg, R.I., and Hakomori, S. (1994) *J. Biol. Chem. 269*, 5644-5652

49. Bremer, E.G., Levery, S.B., Sonnino, S., Ghidoni, R., Caneveri, S., Kannagi, R., and Hakomori, S. (1984) *J. Biol. Chem. 259*, 14773-14777

50. Clausen, H., Levery, S.B., Kannagi, R., and Hakomori, S. (1986) *J. Biol. Chem. 261*, 1380-1387

Table 1. Structures of Glycosphingolipids referred to in this study

Glycosphingolipid	Structure	P Blood Group Activity
CTH	Galα1-4Galβ1-4Glcβ1-1Cer	P^k
Globoside	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	P
Gal-globoside	Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	-
Forssman	GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	-
Globo-H	Fucα1-2Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	-
Globo-A	GalNAcα1-3(Fucα1-2)Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	-
P^1	Galα1-4Galβ1-4GlcNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	P_1
SGG	NeuAcα2-3Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	LKE
DSGG	NeuAcα2-3Galβ1-3(NeuAcα2-6)GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	-

Key: CTH, ceramide trihexoside (Gb$_3$, globotriaosylceramide); globoside, Gb$_4$ (globotetraosylceramide); gal-globoside, Gb$_5$, SSEA3; SGG, sialosyl galactosyl globoside, SSEA4; DSGG, disialosyl galactosyl globoside; Cer, ceramide; Fuc, L-fucose; Gal, D-galactose; Glc, D-glucose; GalNAc, N-acetyl-D-galactosamine; GlcNAc, N-acetyl-D-glucosamine; NeuAc, N-acetylneuraminic acid.

Table 2. Proton chemical shifts (ppm from tetramethylsilane) and $^3J_{1,2}$ coupling constants (Hz) for Sialosylgalactosylgloboside in dimethylsulfoxide-d6/2% D$_2$O at 308°K.

| | NeuAcα2 →3Galβ1 →3GalNAcβ1 →3Galα1 →4Galβ1 →4Glcβ1 →1Cer | | | | | | |
	A	V	IV	III	II	I	R
H-1		4.234	4.579	4.815	4.256	4.185	3.447 (a)
($^3J_{1,2}$)		(7.9)	(8.3)	(4.2)	(7.5)	(7.7)	3.962 (b)
H-2		3.279	3.858	3.766	3.310	3.040	3.781
H-3	2.766 (eq) 1.346 (ax)	3.926	3.675	3.609	3.399		3.880
H-4	3.538	3.706	3.880	3.964	3.815		5.354
H-5				4.117		3.290	5.538
H-6				3.476 (a) 3.454 (b)		3.611 (a) 3.747 (b)	1.935
Nac	1.889		1.795				

FIGURE LEGENDS

Figure 1. HPTLC of ganglioside fractions of normal human kidney separated by HPLC in CMW solvent system 50:40:10. Total ganglioside fraction from human kidney was separated by normal phase preparative HPLC using a linear gradient from IHW 55:40:5 to 55:25:20. Fractions were collected and separated by HPTLC in CMW (50:40:10). Bands were revealed by overlaying HPTLC plate with metabolically [^{35}S]methionine-labeled *E. coli* isolate R45. SGG, sialosyl galactosyl globoside standard; DSGG, disialosyl galactosyl globoside standard. Fractions 39-48, comigrating with SGG standard was pooled and subjected to a second HPLC (semipreparative HPLC, see text). Autoradiograph is shown.

Figure 2. HPTLC of purified gangliosides from human kidney. HPTLC plates of three unknown gangliosides purified from pooled HPLC fraction 53-58 (from semipreparative HPLC). **Panel A,** pooled ganglioside fraction (53-58) previously migrating as a single band in CMW system is resolved into three bands using a solvent system of 1-propanol/water/NH$_4$OH (6:1.5:1) after preparative TLC in the same solvent system; stained with orcinol/sulfuric acid reagent. **Panel B,** the same three gangliosides shown in panel A developed in CMW (50:40:10) and stained with orcinol/sulfuric acid reagent and **Panel C,** autoradiograph of HPTLC plate identical to the samples and solvent conditions used in panel B stained with [^{35}S]-labeled *E. coli* isolate R45. Ganglioside fraction #3 (lane 3) showing strong staining with *E. coli* was structurally characterized.

Figure 3. Selected regions of a 1-D proton NMR spectrum of the monosialosyl ganglioside from human kidney in DMSO-d_6/D$_2$O at 308°K. Region from 1.7-2.1 ppm is attenuated x0.4. Arabic numerals refer to ring protons of residues designated by Roman numerals or capital letters in the corresponding structure drawn at the top of the figure. R refers to protons of the sphingosine backbone only; cis-vinyl and cis-allyl refer to protons of unsaturated *N*-fatty acyl chains; nFA-2 refers to H-2 of non-hydroxylated *N*-fatty acyl chains. Resonances from minor components are designated by small letters and

assigned as follows: a, b, c, d, e: III-1, IV-1, III-4, A-3eq, and B-NAc, respectively, of a monosialoganglioside component proposed to have an isomeric structure with NeuAc (B) linked $\alpha2\rightarrow6$ to β-GalNAc IV of galactosylgloboside; f and h: I-1 and I-2 of ganglioside component with 2-hydroxy fatty acyl chains; g: unknown impurity.

Figure 4. Downfield region of 2-D TOCSY spectrum of the monosialosyl ganglioside from human kidney in DMSO-d_6/D$_2$O at 308°K. Monosaccharide residue H-1 and selected H-5 resonances are designated on diagonal by Roman and Arabic numerals as in Figure 3. Off-diagonal correlations are marked by Arabic numerals only. Upper left section: connectivities originating from V-1 and V-5 (solid lines), R-5 and R-4 (dashed lines), and IV-1 (dashed/dotted lines); lower right section: connectivities originating from III-1 and III-5 (solid lines), I-1 (dashed lines), and II-1 (dashed/dotted lines). Connectivities originating from minor components are shown for III-1, IV-1, and I-1, resonances a, b, and f, respectively in Figure 3.

Figure 5. Positive ion mode ESI-CID-MS (orifice-to-skimmer potential = 180 V) of permethylated monosialosyl ganglioside from human kidney. Panel A, mass range m/z 100-2200; **inset,** expansion of m/z 1000-1550; relevant fragments are designated by nominal monoisotopic mass (see Scheme 1). **Panel B**, mass range m/z 2000-2250 scanned at higher resolution; monosodiated, singly-charged, pseudomolecular ions are designated by fatty acid species (top), nominal monoisotopic mass (parentheses), and measured m/z (to nearest 0.1 u).

Figure 6. Product ion spectra from ESI-MS/CID-MS (orifice-to-skimmer potential = 120 V) of selected doubly-charged disodiated pseudomolecular ions of permethylated monosialosyl ganglioside from human kidney. The orifice-to-skimmer potential was lowered to increase abundance of doubly-charged disodiated pseudomolecular ions. **Panel A**, Q$_1$ selection of m/z 1051.5; **panel B**, Q$_1$ selection of m/z 1065.5; **panel C**, Q$_1$ selection of m/z 1079.5. Relevant fragments are designated by nominal monoisotopic mass (see Scheme 2).
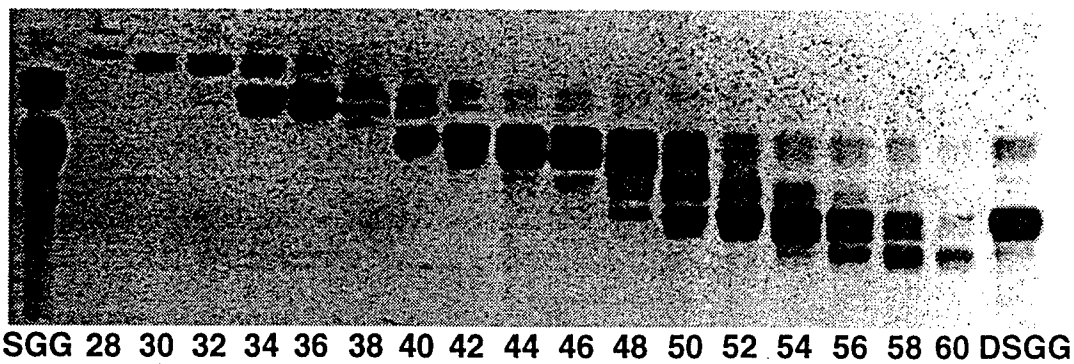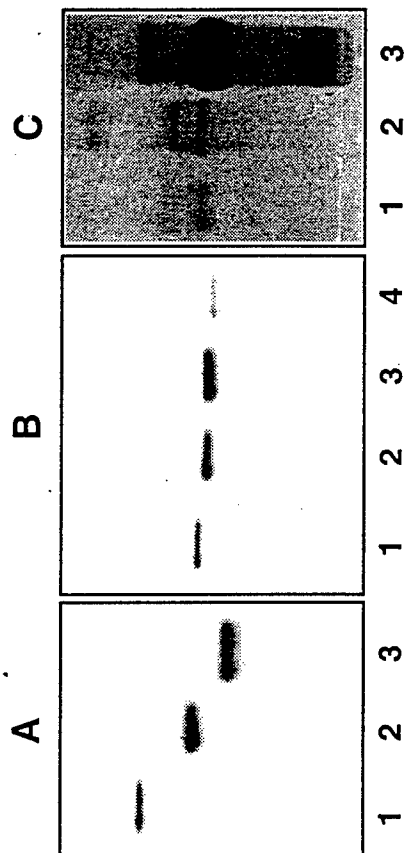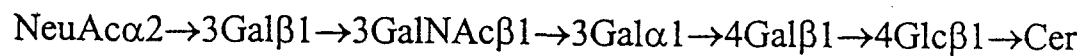
Figure 1

SGG 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 DSGG

Figure 2

Figure 3

NeuAcα2→3Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→Cer

A   V   IV   III   II   I   R

Figure 4

295,000

Figure 5 (Scheme)
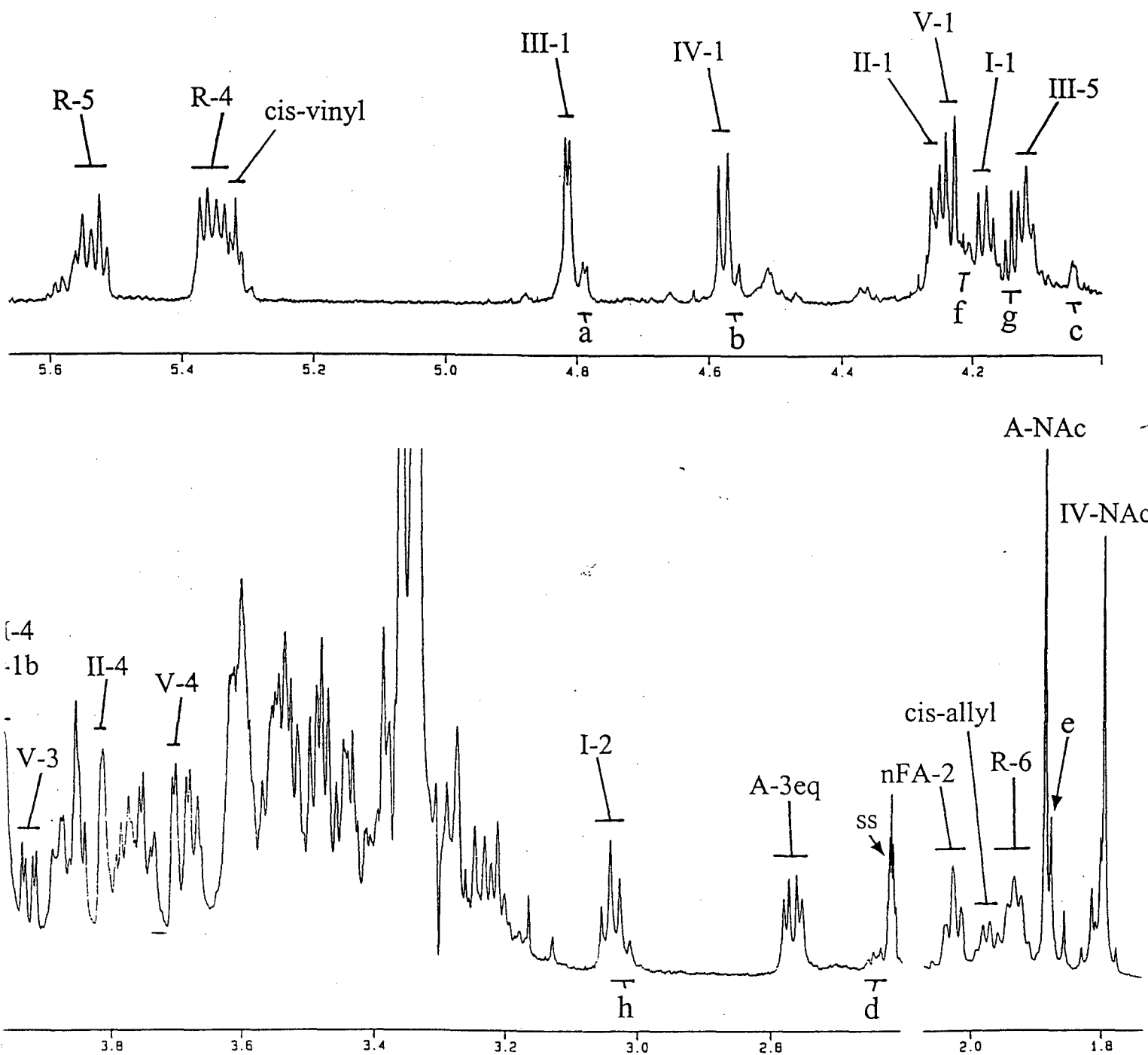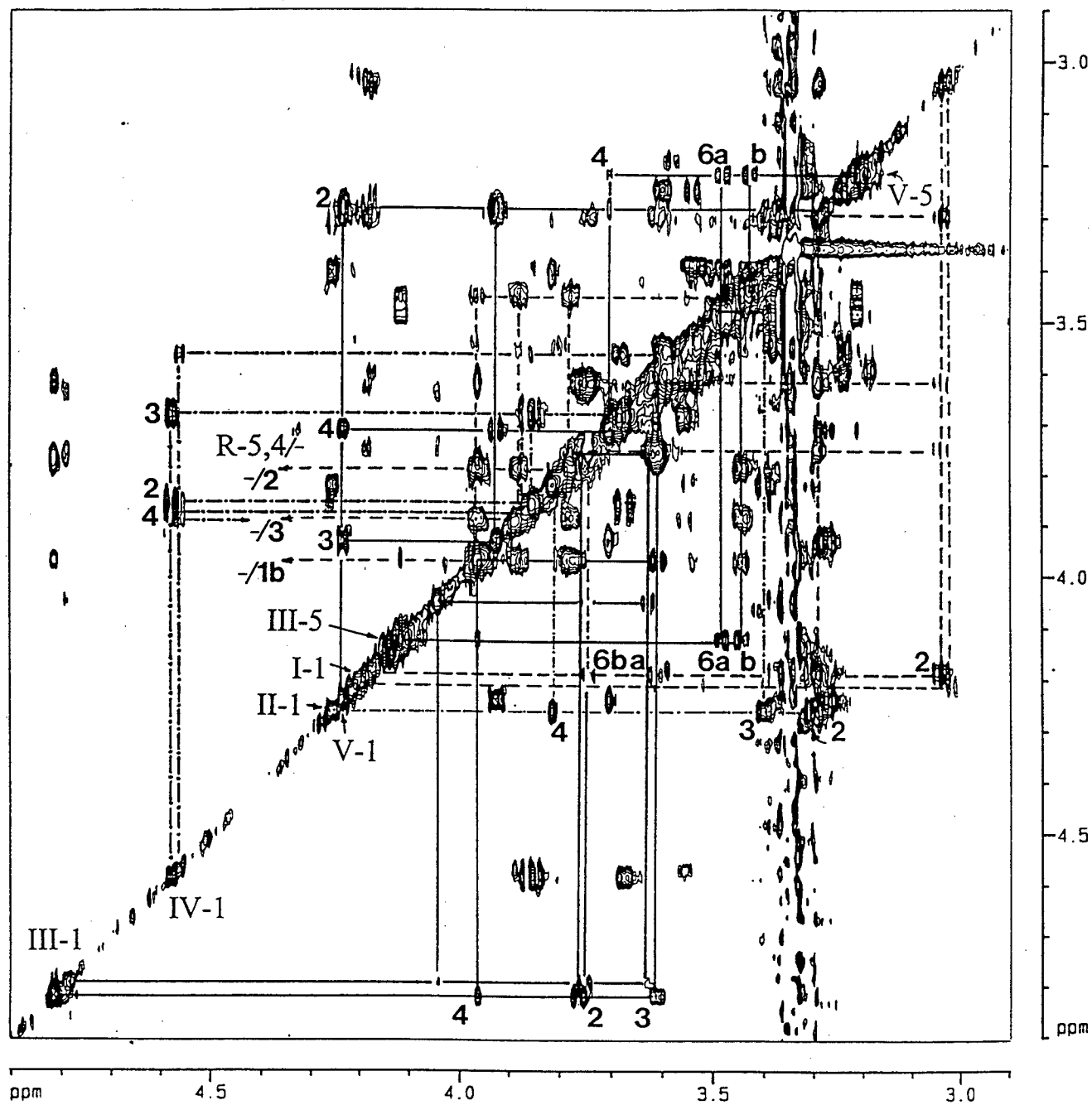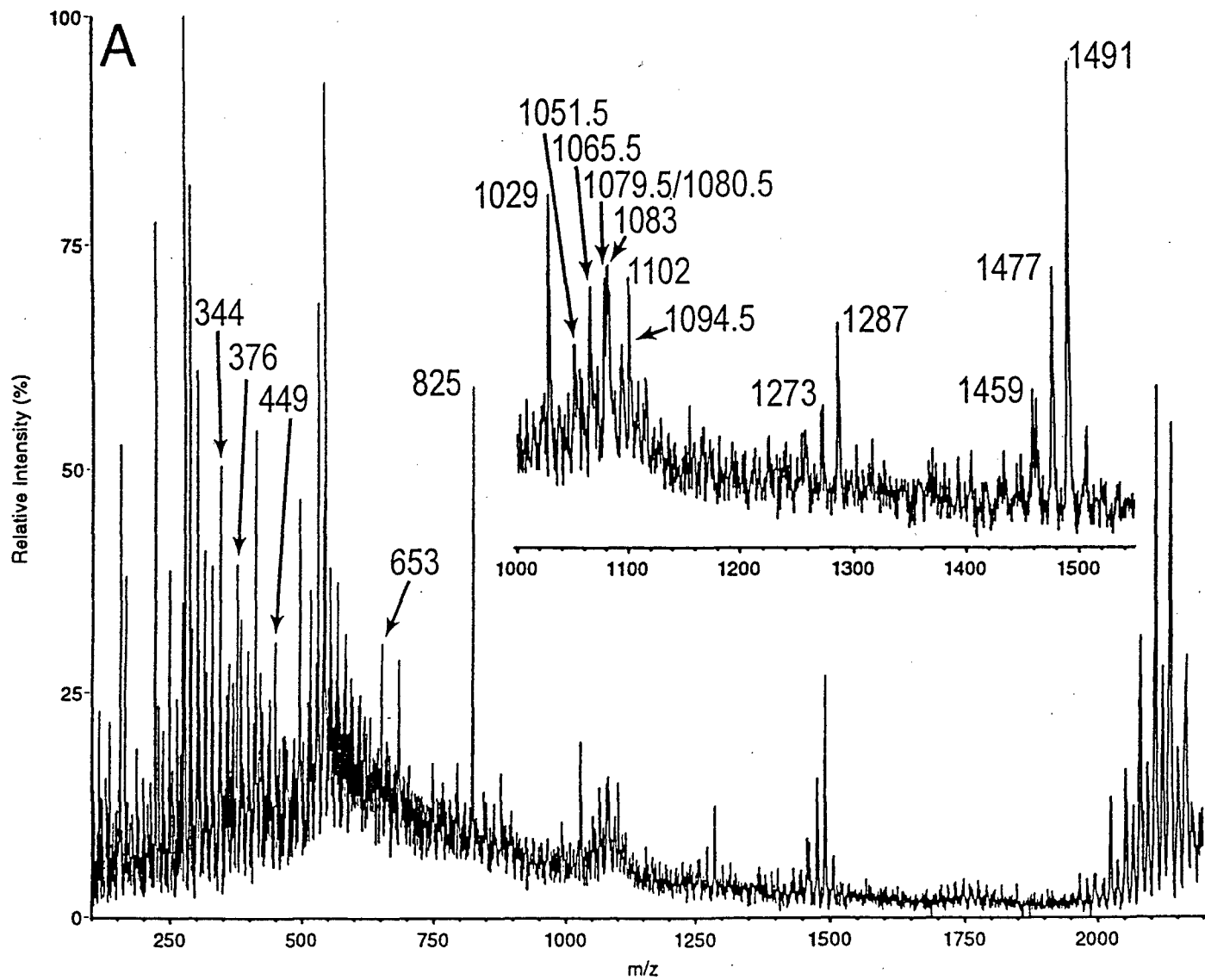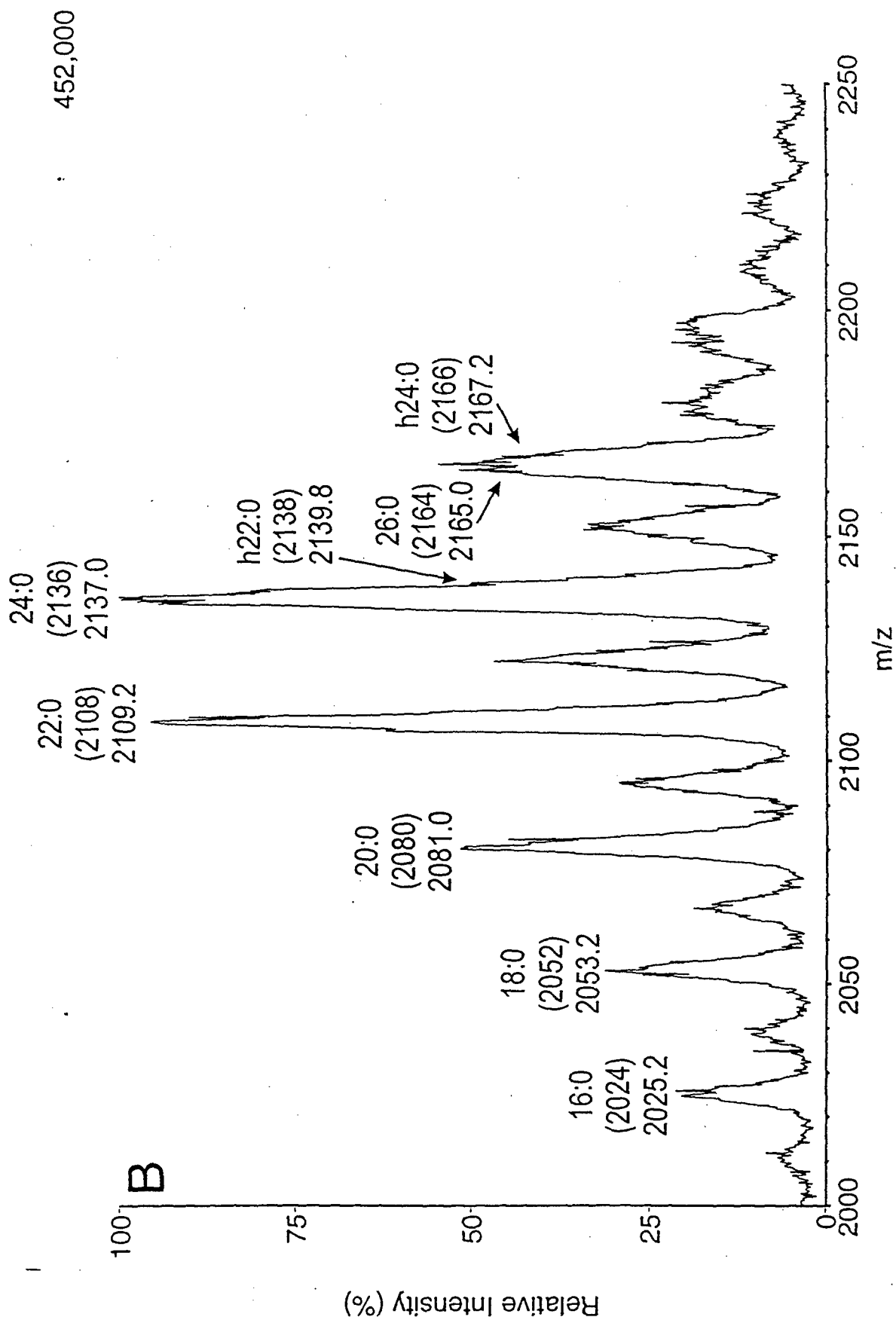
fa	Cer	MW	$M \cdot Na^+$	$M \cdot 2Na^{2+}$
16:0	548	2001	[2024]	[[1023.5]]
18:0	576	2029	[2052]	[[1037.5]]
20:0	604	2057	[2080]	[[1051.5]]
22:0	632	2085	[2108]	[[1065.5]]
24:0	660	2113	[2136]	[[1079.5]]
26:0	688	2141	[2164]	[[1093.5]]
h22:0	662	2115	[2138]	[[1080.5]]
h24:0	690	2143	[2166]	[[1094.5]]

[879]* [1083]*[1287]* [1491]*

$C_{IV} \cdot Na$ $C_{III} \cdot Na$ $C_{II} \cdot Na$ $C_I \cdot Na$

344   [865]   [1069]   [1273]   [1477]

↑ B_{IV} ⌐ B_{III} ⌐   ⌐ $\beta_I \cdot Na$ ⌐

B_{VI} 376   825   1029   [1459]

⌐   ⌐   ⌐   ⌐

NeuAc–O–Hex–O–HexNAc–O–Hex–O–Hex–O–Hex–O–Cer

L   L   L   L   ⌐

[1102]   [898]   [653]   [449]

$Y_V/-$ $Y_{IV}/-$ $Y_{III}/-$ $Y_{II}/C_I \cdot Na$
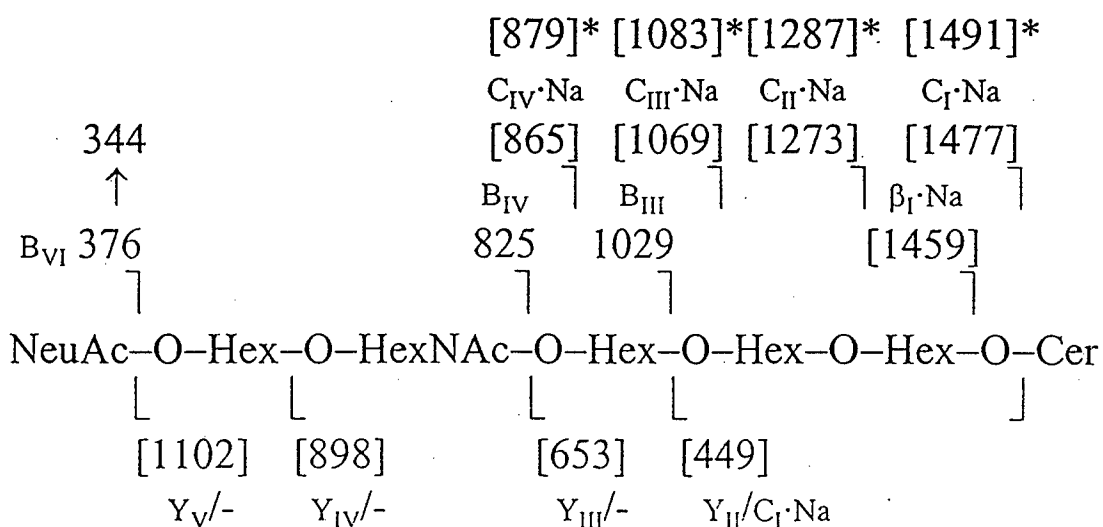
Scheme 1. Prominent pseudomolecular ions and fragmentation of permethylated hexaglycosylceramide in ESI-MS at high orifice-to-skimmer potentials. Key: [ ] = monosodiated monocation; [ ]* = monosodiated monocation + 14 u; [[ ]] = disodiated dication. All values are nominal monoisotopic masses.
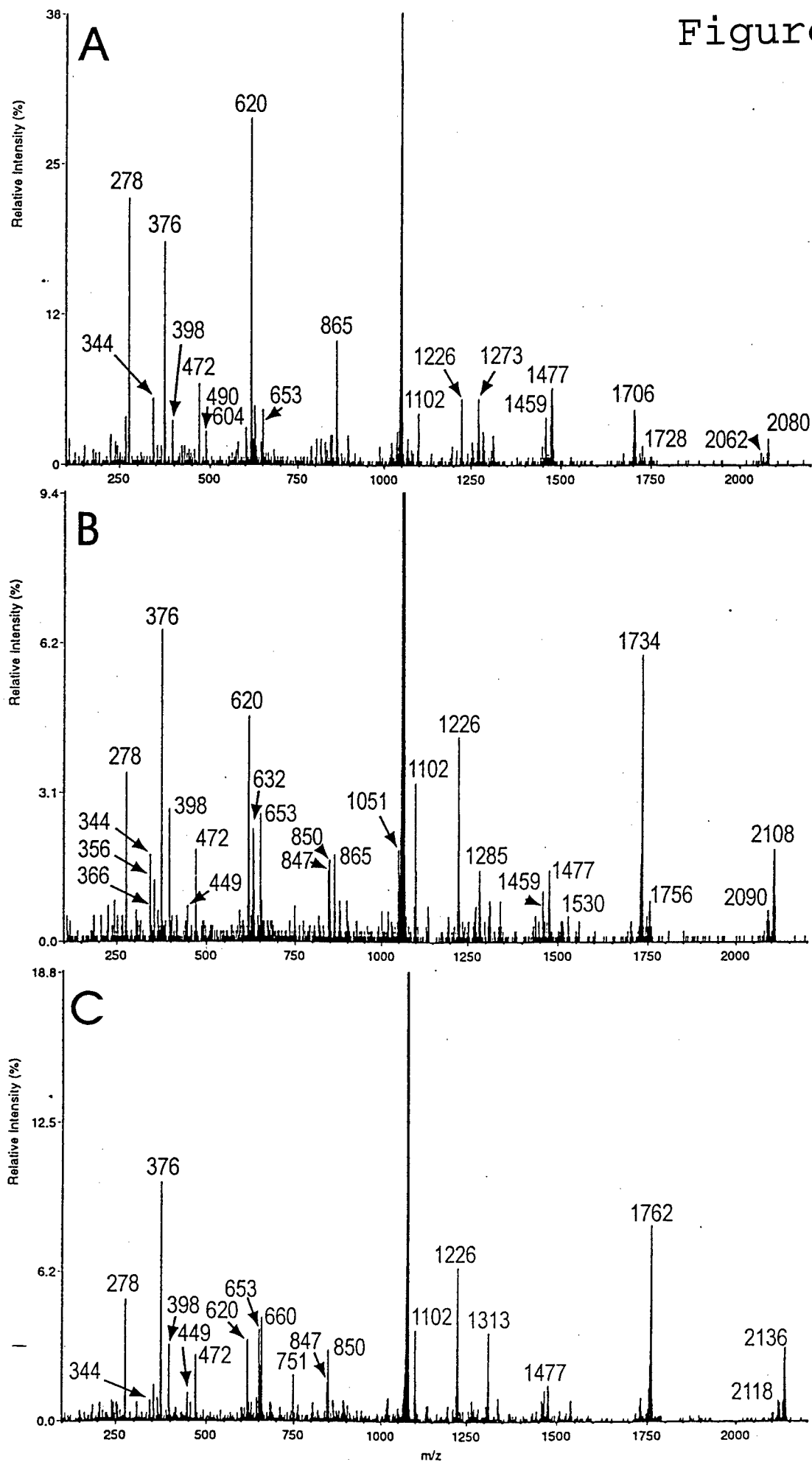
Figure 6

Figure 6 (Scheme)

fa	$M \cdot 2Na^{2+}$	$M \cdot Na^+$	Cer
20:0	[[1051.5]]	[2080]	604
22:0	[[1065.5]]	[2108]	632
24:0	[[1079.5]]	[2136]	660

344
↑
376          $C_{IV} \cdot Na$ $C_{III} \cdot Na$ $C_{II} \cdot Na$ $C_{I} \cdot Na$ Y_0
[366]   [865]   [1069]   [1273]   [1477] 660
↑ $\beta_V \cdot Na$ $\beta_{IV} \cdot Na$ $\beta_{III} \cdot Na$ $\beta_{II} \cdot Na$ $\beta_I \cdot Na$ 632
[398]   [602]   [847]   [1051]   [1255]   [1459] 604
$\beta_{VI} \cdot Na$

NeuAc–O–Hex–O–HexNAc–O–Hex–O–Hex–O–Hex–O–Cer

[472]
$Y_V/\beta_{IV} \cdot Na$

[1102]   [898]   [653]   [449]
$Y_V/-$ $Y_{IV}/-$ $Y_{III}/-$ $Y_{II}/C_I \cdot Na$

[1762]   [1313]
[1734]   [1530]   [1285]
[1706]   [1257]
$Y_V \cdot Na$ $Y_{IV} \cdot Na$ $Y_{III} \cdot Na$

Scheme 2. Fragmentation of Q_1-selected doubly charged disodiated pseudomolecular ions $M \cdot 2Na^{2+}$ of permethylated hexaglycosyl-ceramide in ESI-MS/CID-MS. Key: [ ] = monosodiated monocation. All values are nominal monoisotopic masses.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)   21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
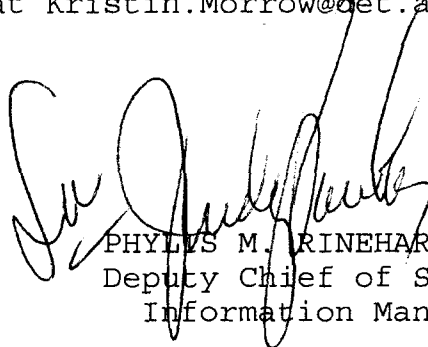VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has
reexamined the need for the limitation assigned to technical
reports written for this Command. Request the limited
distribution statement for the enclosed accession numbers be
changed to "Approved for public release; distribution unlimited."
These reports should be released to the National Technical
Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at
DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl   PHYLIS M. RINEHART
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